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=> file medline biosis

COST IN U.S. DOLLARS                                    SINCE FILE      TOTAL
                                                           ENTRY    SESSION
FULL ESTIMATED COST                                         0.15       0.15

FILE 'MEDLINE' ENTERED AT 10:27:12 ON 03 MAY 2000

FILE 'BIOSIS' ENTERED AT 10:27:12 ON 03 MAY 2000
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=> s reporter

L1       35925 REPORTER

=> s synergism or coperat?

L2       50093 SYNERGISM OR COPERAT?

=> s cooperat?

L3       87345 COOPERAT?

=> s synergi?

L4       98277 SYNERGI?

=> s l3 or l4

L5      183579 L3 OR L4

=> s amplif?

L6      131206 AMPLIF?

=> s l3 or l4

L7      183579 L3 OR L4

=> s l7 and l1

L8        1582 L7 AND L1

=> s l8 and l6

L9          38 L8 AND L6

=> dup rem l9

PROCESSING COMPLETED FOR L9
L10          28 DUP REM L9 (10 DUPLICATES REMOVED)

=> s l8 and cotransfect?

L11        201 L8 AND COTRANSFECT?

=> s l11 and l6

L12         10 L11 AND L6

=> dup rem l10
```

PROCESSING COMPLETED FOR L[redacted]
L13 28 DUP REM L1[redacted]0 DUPLICATES REMOVED)

=> dup rem l12\

ENTER L# LIST OR (END):end

=> dup rem l12

PROCESSING COMPLETED FOR L12
L14 7 DUP REM L12 (3 DUPLICATES REMOVED)

=> l10 not l14

L10 IS NOT A RECOGNIZED COMMAND
The previous command name entered was not recognized by the system.
For a list of commands available to you in the current file, enter
"HELP COMMANDS" at an arrow prompt (=>).

=> s l10 not l14

L15 21 L10 NOT L14

=> d ibib abs l14

L14 ANSWER 1 OF 7 BIOSIS COPYRIGHT 2000 BIOSIS
ACCESSION NUMBER: 2000:113584 BIOSIS
DOCUMENT NUMBER: PREV200000113584
TITLE: The human immunodeficiency virus type 1 Tat protein up-regulates the promoter activity of the beta-chemokine monocyte chemoattractant protein 1 in the human astrocytoma cell line U-87 MG: Role of SP-1, AP-1, and NF-kappaB consensus sites.
AUTHOR(S): Lim, Siew Pheng; Garzino-Demo, Alfredo (1)
CORPORATE SOURCE: (1) Institute of Human Virology, University of Maryland Biotechnology Institute, 725 W. Lombard St., Baltimore, MD, 21201-1192 USA
SOURCE: Journal of Virology, (Feb., 2000) Vol. 74, No. 4, pp. 1632-1640.
ISSN: 0022-538X.
DOCUMENT TYPE: Article
LANGUAGE: English
SUMMARY LANGUAGE: English
AB It has been shown that the human immunodeficiency virus type 1 (HIV-1) Tat protein can specifically enhance expression and release of monocyte chemoattractant protein 1 (MCP-1) from human astrocytes. In this study, we show evidence that Tat-induced MCP-1 expression is mediated at the transcriptional level. Transient transfection of an expression construct encoding the full-length Tat into the human glioblastoma-astrocytoma cell line U-87 MG enhances **reporter** gene activity from **cotransfected** deletion constructs of the MCP-1 promoter. HIV-1 Tat exerts its effect through a minimal construct containing 213 nucleotides upstream of the translational start site. Site-directed mutagenesis studies indicate that an SP1 site (located between nucleotides -123 and -115) is critical for both constitutive and Tat-enhanced expression of the human MCP-1 promoter, as mutation of this SP1 site significantly diminished **reporter** gene expression in both instances. Gel retardation experiments further demonstrate that Tat strongly enhances the binding of SP1 protein to its DNA element on the MCP-1 promoter. Moreover, we also observe an increase in the binding activities of transcriptional

factors AP1 and NF-kappaB to the MCP-1 promoter following Tat treatment. Mutagenesis studies show that an upstream AP1 site and adjacent NF-kappaB site (located at -128 to -122 and -150 to -137, respectively) play a role in Tat-mediated transactivation. In contrast, a further upstream AP1 site (-156 to -150) does not appear to be crucial for promoter activity. We postulate that a Tat-mediated increase in SP1 binding activities augments the binding of AP1 and NF-kappaB, leading to **synergistic** activation of the MCP-1 promoter.

=> d his

(FILE 'HOME' ENTERED AT 10:26:58 ON 03 MAY 2000)

FILE 'MEDLINE, BIOSIS' ENTERED AT 10:27:12 ON 03 MAY 2000

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L1         35925 S REPORTER
L2         50093 S SYNERGISM OR COPERAT?
L3         87345 S COOPERAT?
L4         98277 S SYNERGI?
L5        183579 S L3 OR L4
L6        131206 S AMPLIF?
L7        183579 S L3 OR L4
L8          1582 S L7 AND L1
L9            38 S L8 AND L6
L10           28 DUP REM L9 (10 DUPLICATES REMOVED)
L11          201 S L8 AND COTRANSFECT?
L12           10 S L11 AND L6
L13           28 DUP REM L10 (0 DUPLICATES REMOVED)
L14            7 DUP REM L12 (3 DUPLICATES REMOVED)
L15           21 S L10 NOT L14
```

=> d ibib abs 1-7 l14

L14 ANSWER 1 OF 7 BIOSIS COPYRIGHT 2000 BIOSIS

ACCESSION NUMBER: 2000:113584 BIOSIS
DOCUMENT NUMBER: PREV200000113584
TITLE: The human immunodeficiency virus type 1 Tat protein up-regulates the promoter activity of the beta-chemokine monocyte chemoattractant protein 1 in the human astrocytoma cell line U-87 MG: Role of SP-1, AP-1, and NF-kappaB consensus sites.
AUTHOR(S): Lim, Siew Pheng; Garzino-Demo, Alfredo (1)
CORPORATE SOURCE: (1) Institute of Human Virology, University of Maryland Biotechnology Institute, 725 W. Lombard St., Baltimore, MD, 21201-1192 USA
SOURCE: Journal of Virology, (Feb., 2000) Vol. 74, No. 4, pp. 1632-1640.
ISSN: 0022-538X.
DOCUMENT TYPE: Article
LANGUAGE: English
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human MCP-1 promoter, mutation of this SP1 site significantly diminished **reporter** gene expression in both instances. Gel retardation experiments further demonstrate that Tat strongly enhances the binding of SP1 protein to its DNA element on the MCP-1 promoter. Moreover, we also observe an increase in the binding activities of transcriptional factors AP1 and NF-kappaB to the MCP-1 promoter following Tat treatment. Mutagenesis studies show that an upstream AP1 site and an adjacent NF-kappaB site (located at -128 to -122 and -150 to -137, respectively) play a role in Tat-mediated transactivation. In contrast, a further upstream AP1 site (-156 to -150) does not appear to be crucial for promoter activity. We postulate that a Tat-mediated increase in SP1 binding activities augments the binding of AP1 and NF-kappaB, leading to **synergistic** activation of the MCP-1 promoter.

L14 ANSWER 2 OF 7 BIOSIS COPYRIGHT 2000 BIOSIS
ACCESSION NUMBER: 1998:495811 BIOSIS
DOCUMENT NUMBER: PREV199800495811
TITLE: Transcriptional regulation of mouse mu-opioid receptor gene.
AUTHOR(S): Ko, Jane L. (1); Liu, Hsien-Ching; Minnerath, Sharon R.; Loh, Horace H.
CORPORATE SOURCE: (1) Dep. Pharmacol., Univ. Minnesota Med. Sch., 3-249 Millard Hall, 435 Delaware St. SE, Minneapolis, MN 55455 USA
SOURCE: Journal of Biological Chemistry, (Oct. 16, 1998) Vol. 273, No. 42, pp. 27678-27685.
ISSN: 0021-9258.
DOCUMENT TYPE: Article
LANGUAGE: English
AB Previously, the existence of dual promoters was reported in mouse mu-opioid receptor (mor) gene, with mor transcription in the mouse brain predominantly initiated by the proximal promoter. In this study, we further analyzed the proximal promoter region, base pairs -450 to -249, to identify cis-DNA regulatory elements and trans-acting protein factors that are important for mor promoter activity. The results revealed that a mor inverted GA (iGA) motif and a canonical Sp1 binding site are required for the promoter activity. Using electrophoretic mobility shift analysis, we identified nuclear proteins that specifically bind to the mor iGA motif and that are immunologically related to Sp1 and Sp3. Mutation of the mor iGA motif, resulting in a loss of Sp binding, led to a 50% decrease in activity. Mutation of the canonical Sp1 binding site yielded a lesser (approximately 25%) loss of activity. Mutation of both motifs together resulted in an approximately 70% decrease in activity. In **cotransfection** assays using Drosophila SL2 cells, Sp1 trans-activated the promoter in a manner dependent on the presence of mor iGA and canonical Sp1 binding motifs. Sp3 can also trans-activate the promoter, and furthermore, Sp1 and Sp3 can trans-activate the mor promoter additively. Our results suggest that combined or **cooperative** interaction of Sp transcription factors within the proximal promoter is necessary for activation of mor gene transcription.

L14 ANSWER 3 OF 7 MEDLINE
ACCESSION NUMBER: 97299670 MEDLINE
DOCUMENT NUMBER: 97299670
TITLE: The c-Jun-induced transformation process involves complex regulation of tenascin-C expression.
AUTHOR: Mettouchi A; Cabon F; Montreau N; Dejong V; Vernier P; Gherzi R; Mercier G; Binetruy B
CORPORATE SOURCE: Institut de Recherche sur le Cancer, CNRS UPR9079, Villejuif, France.
SOURCE: MOLECULAR AND CELLULAR BIOLOGY, (1997 Jun) 17 (6) 3202-9.
Journal code: NGY. ISSN: 0270-7306.
PUB. COUNTRY: United States

Journal Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199708

AB In **cooperation** with an activated ras oncogene, the site-dependent AP-1 transcription factor c-Jun transforms primary rat embryo fibroblasts (REF). Although signal transduction pathways leading to activation of c-Jun proteins have been extensively studied, little is known about c-Jun cellular targets. We identified c-Jun-upregulated cDNA clones homologous to the tenascin-C gene by differential screening of a cDNA library from REF. This tightly regulated gene encodes a rare extracellular matrix protein involved in cell attachment and migration and in the control of cell growth. Transient overexpression of c-Jun induced tenascin-C expression in primary REF and in FR3T3, an established fibroblast cell line. Surprisingly, tenascin-C synthesis was repressed after stable transformation by c-Jun compared to that in the nontransformed parental cells. As assessed by using the tenascin-C (-220 to +79) promoter fragment cloned in a **reporter** construct, the c-Jun-induced transient activation is mediated by two binding sites: one GCN4/AP-1-like site, at position -146, and one NF-kappaB site, at position -210. Furthermore, as demonstrated by gel shift experiments and **cotransfections** of the **reporter** plasmid and expression vectors encoding the p65 subunit of NF-kappaB and c-Jun, the two transcription factors bind and **synergistically** transactivate the tenascin-C promoter. We previously described two other extracellular matrix proteins, SPARC and thrombospondin-1, as c-Jun targets. Thus, our results strongly suggest that the regulation of the extracellular matrix composition plays a central role in c-Jun-induced transformation.

L14 ANSWER 4 OF 7 MEDLINE DUPLICATE 1
ACCESSION NUMBER: 97254492 MEDLINE
DOCUMENT NUMBER: 97254492
TITLE: Transcriptional regulation of the human 'leukemia inhibitory factor' gene: modulation by glucocorticoids and estradiol.
AUTHOR: Bamberger A M; Erdmann I; Bamberger C M; Jenatschke S S; Schulte H M
CORPORATE SOURCE: IHF, Institute for Hormone and Fertility Research, University of Hamburg, Germany.
SOURCE: MOLECULAR AND CELLULAR ENDOCRINOLOGY, (1997 Mar 14) 127 (1) 71-9.
Journal code: E69. ISSN: 0303-7207.
PUB. COUNTRY: Ireland
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199708
ENTRY WEEK: 19970803

AB Leukemia inhibitory factor (LIF) is a pleiotropic cytokine implicated in various pathological conditions, such as rheumatoid arthritis and osteoporosis. Despite its possible importance as a therapeutic target, very little is known about the regulation of human LIF. In particular, its regulation at the promoter level has not been studied so far, and was, therefore, the focus of the present work. After showing that Jurkat T lymphoma cells can be induced to express endogenous LIF mRNA, we used this cell line as a model to study the regulation of the human LIF promoter in transient transfection assays. For this purpose, a 666 bp fragment of the human LIF 5'-flanking region, **amplified** from genomic DNA by nested polymerase chain reaction (PCR), was used for the construction of a luciferase **reporter** plasmid (hLIF666-Luc). In unstimulated Jurkat cells, the human LIF promoter showed low constitutive activity. The

promoter was induced upon stimulation with phorbol ester (TPA). Combined stimulation with TPA and the calcium ionophore ionomycin resulted in strong **synergistic** induction of luciferase activity from the LIF promoter. Transfection experiments with deletion constructs (hLIF274-Luc and hLIF82-Luc) located the region required for this induction to a 192 bp portion of the promoter, which carries two putative c-ets binding sites. We then investigated the effect of glucocorticoids and estradiol by **cotransfecting** the respective receptors. Both hormones strongly inhibited the stimulation of the LIF promoter by TPA and ionomycin. Since LIF is implicated in the pathogenesis of inflammatory and degenerative conditions, such as rheumatoid arthritis and osteoporosis, the finding that therapeutic agents employed in the treatment of such conditions, i.e. glucocorticoids and estrogens, can modulate the induction of LIF at the transcriptional level, is of particular interest.

L14 ANSWER 5 OF 7 MEDLINE DUPLICATE 2
ACCESSION NUMBER: 96413346 MEDLINE
DOCUMENT NUMBER: 96413346
TITLE: YY1 and NF1 both activate the human p53 promoter by alternatively binding to a composite element, and YY1 and E1A **cooperate** to **amplify** p53 promoter activity.
AUTHOR: Furlong E E; Rein T; Martin F
CORPORATE SOURCE: Pharmacology Department, University College Dublin, Ireland.
SOURCE: MOLECULAR AND CELLULAR BIOLOGY, (1996 Oct) 16 (10) 5933-45.
Journal code: NGY. ISSN: 0270-7306.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199701

AB A novel transcription factor binding element in the human p53 gene promoter has been characterized. It lies about 100 bp upstream of the major reported start site for human p53 gene transcription. On the basis of DNase I footprinting studies, electromobility shift assay patterns, sequence specificity of binding, the binding pattern of purified transcription factors, effects of specific antibodies, and methylation interference analysis we have identified the site as a composite element which can bind both YY1 and NF1 in an independent and mutually exclusive manner. The site is conserved in the human, rat, and mouse p53 promoters. The occupancy of the site varies in a tissue-specific manner. It binds principally YY1 in nuclear extracts of rat testis and spleen and NF1 in extracts of liver and prostate. This may facilitate tissue-specific control of p53 gene expression. When HeLa cells were transiently transfected with human p53 promoter-chloramphenicol acetyltransferase **reporter** constructs, a mutation in this composite element which disabled YY1 and NF1 binding caused a mean 64% reduction in basal p53 promoter activity. From mutations which selectively impaired YY1 or NF1 binding and the overexpression of YY1 or NF1 in HeLa cells we concluded that both YY1 and NF1 function as activators when bound to this site. In transient **cotransfections** E1A could induce the activity of the p53 promoter to a high level; 12S E1A was threefold as efficient as 13S E1A in this activity, and YY1 bound to the composite element was shown to mediate 55% of this induction. Overexpressed YY1 was shown to be able to **synergistically** activate the p53 promoter with E1A when not specifically bound to DNA. Deletion of an N-terminal domain of E1A, known to be required for direct E1A-YY1 interaction and E1A effects mediated through transcriptional activator p300, blocked the E1A induction of p53 promoter activity.

L14 ANSWER 6 OF 7 BIOSIS COPYRIGHT 2000 BIOSIS
ACCESSION NUMBER: 1995:407480 BIOSIS
DOCUMENT NUMBER: PREV199598421780
TITLE: Pituitary-type transcription of the human prolactin gene in

the absence of pit-1.
AUTHOR(S): Gellersen, Birgit (1); Kempf, Rita (1); Helgmann, Ralph (1); Dimattia, Gabriel E.
CORPORATE SOURCE: (1) Inst. Hormone Fertility Res., Univ. Hamburg, 22529 Hamburg Germany
SOURCE: Molecular Endocrinology, (1995) Vol. 9, No. 7, pp. 887-901.
ISSN: 0888-8809.
DOCUMENT TYPE: Article
LANGUAGE: English

AB We describe a human (h) PRL-producing cell line, SKUT-1B-20, which we isolated as a subclone of a uterine sarcoma cell line. Although this cell line is of uterine origin, it does not use the decidual-specific upstream promoter of the hPRL gene, but transcribes the hPRL gene from the downstream pituitary-type transcription start site, as determined by Northern blot, reverse transcriptase-polymerase chain reaction and primer extension analyses. This is particularly intriguing because SKUT-1B-20 cells lack the transcription factor Pit-1. No Pit-1 messenger RNA was detectable by reverse transcriptase-polymerase chain reaction, and endogenous Pit-1 target genes (GH, PRL, and Pit-1) were refractory to transfected Pit-1 expression vector, whereas in **cotransfection** experiments, Pit-1 efficiently activated **reporter** gene fusion constructs carrying 5'-flanking sequences of the human and rat PRL or the mouse Pit-1 genes. By transfecting **reporter** genes containing 8.7 kilobases of DNA flanking the hPRL pituitary-specific start site (hPRL-8700/Luc) and deletions thereof, we located a Pit-1-independent cis-active region more than 7 kilobases upstream of the start site. The most distal 1650 or 880 base pairs of the hPRL genomic fragment (which extends to -8784 base pairs), when placed directly upstream of the homologous hPRL or the heterologous thymidine kinase promoters, conferred transcriptional activation to those promoters. SKUT-1B-20 cell-specific activation of hPRL-8700/Luc could not be suppressed by the introduction of an inhibitor of protein kinase A (PKA), PKI. This is the first demonstration of pituitary-type PRL gene transcription independent of Pit-1 and activation of the PKA pathway. The SKUT-1B-20 cell line was then used in reconstitution experiments to delineate the role of Pit-1 in modulating the transcriptional effects of phorbol ester, PKA, and estrogen receptor (ER) on the hPRL gene. The low response of hPRL/luciferase fusion genes to phorbol ester was greatly enhanced by **cotransfected** Pit-1 and was mediated by the proximal region between -250 and -38. The catalytic subunit of PKA, C-beta, was able to elicit a moderate induction of hPRL-8700/Luc even in the absence of Pit-1; the response was strongly **amplified** by coexpression of Pit-1. A potential estrogen response element has been located in the hPRL gene sequence at a position similar to that of the estrogen response element of the rat PRL gene immediately adjacent to the distal enhancer. In striking contrast to the dramatic **cooperative** action of ER and Pit-1 on the rat PRL gene, which has been reported previously and was also supported by SKUT-1B-20 cells, no such response was obtained with hPRL gene constructs. Liganded ER caused a mere 2-fold induction of **reporter** gene activity regardless of the absence or presence of Pit-1. This demonstration of a functional dissimilarity between rat and hPRL gene regulatory regions is congruous with the different roles of estrogen in lactotrope control in the two species.

L14 ANSWER 7 OF 7 MEDLINE DUPLICATE 3
ACCESSION NUMBER: 90279073 MEDLINE
DOCUMENT NUMBER: 90279073
TITLE: Epstein-Barr virus nuclear antigen 2 transactivates latent membrane protein LMP1.
AUTHOR: Wang F; Tsang S F; Kurilla M G; Cohen J I; Kieff E
CORPORATE SOURCE: Department of Medicine, Harvard Medical School, Boston, Massachusetts 02115.
CONTRACT NUMBER: CA17006 (NCI)

CA0139[redacted](NCI)
CA0141[redacted](NCI)
+
SOURCE: JOURNAL OF VIROLOGY, (1990 Jul) 64 (7) 3407-16.
Journal code: KCV. ISSN: 0022-538X.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals; Cancer Journals
ENTRY MONTH: 199009

AB Several lines of evidence are compatible with the hypothesis that Epstein-Barr virus (EBV) nuclear antigen 2 (EBNA-2) or leader protein (EBNA-LP) affects expression of the EBV latent infection membrane protein LMP1. We now demonstrate the following. (i) Acute transfection and expression of EBNA-2 under control of simian virus 40 or Moloney murine leukemia virus promoters resulted in increased LMP1 expression in P3HR-1-infected Burkitt's lymphoma cells and the P3HR-1 or Daudi cell line. (ii) Transfection and expression of EBNA-LP alone had no effect on LMP1 expression and did not act **synergistically** with EBNA-2 to affect LMP1 expression. (iii) LMP1 expression in Daudi and P3HR-1-infected cells was controlled at the mRNA level, and EBNA-2 expression in Daudi cells increased LMP1 mRNA. (iv) No other EBV genes were required for EBNA-2 transactivation of LMP1 since **cotransfection** of recombinant EBNA-2 expression vectors and genomic LMP1 DNA fragments enhanced LMP1 expression in the EBV-negative B-lymphoma cell lines BJAB, Loucke, and BL30. (v) An EBNA-2-responsive element was found within the -512 to +40 LMP1 DNA since this DNA linked to a chloramphenicol acetyltransferase **reporter** gene was transactivated by **cotransfection** with an EBNA-2 expression vector. (vi) The EBV type 2 EBNA-2 transactivated LMP1 as well as the EBV type 1 EBNA-2. (vii) Two deletions within the EBNA-2 gene which rendered EBV transformation incompetent did not transactivate LMP1, whereas a transformation-competent EBNA-2 deletion mutant did transactivate LMP1. LMP1 is a potent effector of B-lymphocyte activation and can act **synergistically** with EBNA-2 to induce cellular CD23 gene expression. Thus, EBNA-2 transactivation of LMP1 **amplifies** the biological impact of EBNA-2 and underscores its central role in EBV-induced growth transformation.

=> d his

(FILE 'HOME' ENTERED AT 10:26:58 ON 03 MAY 2000)

FILE 'MEDLINE, BIOSIS' ENTERED AT 10:27:12 ON 03 MAY 2000
L1 35925 S REPORTER
L2 50093 S SYNERGISM OR COPERAT?
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L12 10 S L11 AND L6
L13 28 DUP REM L10 (0 DUPLICATES REMOVED)
L14 7 DUP REM L12 (3 DUPLICATES REMOVED)
L15 21 S L10 NOT L14

=> d ibib abs l15 1-21

L15 ANSWER 1 OF 21 MEDLINE
ACCESSION NUMBER: 2000105561 MEDLINE

DOCUMENT NUMBER: 201055
TITLE: Rapid antibody responses by low-dose, single-step, dendritic cell-targeted immunization.
AUTHOR: Wang H; Griffiths M N; Burton D R; Ghazal P
CORPORATE SOURCE: Department of Immunology, Division of Virology, Scripps Research Institute, La Jolla, CA 92037, USA.
CONTRACT NUMBER: AI 33292 (NIAID)
AI 39808 (NIAID)
T32 AI07266-16 (NIAID)
SOURCE: PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, (2000 Jan 18) 97 (2) 847-52. Journal code: PV3. ISSN: 0027-8424.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals; Cancer Journals
ENTRY MONTH: 200005
ENTRY WEEK: 20000501
AB We have compared the kinetics of antibody responses in conventional and dendritic cell-targeted immunization by using a model antigen in mice. Targeting was achieved by linking the **reporter** antigen (polyclonal goat anti-hamster antibody) to N418, a hamster mAb that binds to the CD11c molecule on the surface of murine dendritic cells. Intradermal injection of submicrogram quantities of goat anti-hamster antibody complexed to mAb N418 elicited goat antibody-specific serum IgG in mice. Antigen-specific IgG titers were detectable by day 5, with titers that ranged from 1:1000 to 1:100,000 by day 7. In contrast, when the goat antigen was injected alone or in the presence of a hamster antibody control to form nontargeted complexes, goat-specific serum IgG was undetectable at day 7. Additional control experiments showed that the interaction between the model antigen and mAb N418 is required for **amplification** of the serum antibody response. These studies demonstrate that a single-step, facilitated-delivery of small amounts of protein antigen to dendritic cells in vivo can give very rapid and high antibody responses. The approach may be particularly useful for vaccination immediately before or just after exposure to a pathogen and may enhance the utility of subunit antigens as immunogens.

L15 ANSWER 2 OF 21 MEDLINE
ACCESSION NUMBER: 1999190417 MEDLINE
DOCUMENT NUMBER: 99190417
TITLE: Concerted expression of BK virus large T- and small t-antigens strongly enhances oestrogen receptor-mediated transcription.
AUTHOR: Moens U; Van Ghelue M; Johansen B; Seternes O M
CORPORATE SOURCE: Department of Gene Biology, Institute of Medical Biology, University of Tromso, Norway.. ugom@fagmed.uit.no
SOURCE: JOURNAL OF GENERAL VIROLOGY, (1999 Mar) 80 (Pt 3) 585-94. Journal code: I9B. ISSN: 0022-1317.
PUB. COUNTRY: ENGLAND: United Kingdom
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals; Cancer Journals
ENTRY MONTH: 199906
ENTRY WEEK: 19990603
AB Previous studies have shown that the human polyomavirus BK (BKV) genome contains an oestrogen response element (ERE). This isolated element binds its cognate receptor in vitro and can mediate 17beta-oestradiol-induced gene expression when linked to a heterologous promoter. The roles of the ERE- and the AP-1-binding sites in oestrogen receptor-directed transcription from the complete BKV promoter/enhancer (Dunlop strain) have been examined and the effects of the general co-activator CBP and large T- and small t-antigens on oestrogen receptor-mediated transcription have been investigated. A constitutive activated oestrogen receptor stimulated BKV promoter activity in HeLa cells. Mutations in either the ERE- or the AP-1-binding sites did not impair oestrogen receptor-induced activation of

the BKV Dunlop promot[redacted] while mutations in both bindi[redacted] motifs almost completely abolished [redacted]strogen receptor-induced transc[redacted]ption. Simultaneous expression of large T- and small t-antigens strongly activated oestrogen receptor-mediated transcription. When expressed separately, only large T-antigen moderately stimulated oestrogen receptor-mediated transcription. The stimulatory effect of large T-antigen on the activity of the oestrogen receptor is probably indirect because no physical interaction between the two proteins was detected in a two-hybrid assay. Large T-antigen abrogated the **synergistic** effect on transcription between this nuclear receptor and the general co-activator CBP. The findings that the BKV early proteins **amplify** oestrogen receptor-mediated transcription may have important biological implications in individuals with raised oestrogen concentrations.

L15 ANSWER 3 OF 21 MEDLINE
ACCESSION NUMBER: 1999101939 MEDLINE
DOCUMENT NUMBER: 99101939
TITLE: Concerted regulation of low density lipoprotein receptor gene expression by follicle-stimulating hormone and insulin-like growth factor I in porcine granulosa cells: promoter activation, messenger ribonucleic acid stability, and sterol feedback.
AUTHOR: LaVoie H A; Garmey J C; Day R N; Veldhuis J D
CORPORATE SOURCE: Department of Internal Medicine, University of Virginia Health Sciences Center, Charlottesville 22908, USA.. hlavoie@maine.maine.edu
CONTRACT NUMBER: HD-07382 (NICHD)
HD-08019 (NICHD)
HD-16393 (NICHD)
+
SOURCE: ENDOCRINOLOGY, (1999 Jan) 140 (1) 178-86.
Journal code: EGZ. ISSN: 0013-7227.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Abridged Index Medicus Journals; Priority Journals; Cancer Journals
ENTRY MONTH: 199903
ENTRY WEEK: 19990305
AB Insulin-like growth factor I (IGF-I) and the gonadotropin, FSH, can **synergize** to stimulate progesterone production in primary cultures of maturing granulosa cells. These trophic hormones increase low density lipoprotein (LDL) receptor binding and internalization, and the utilization of LDL-borne cholesterol by granulosa cells. To determine whether and how IGF-I and FSH control the genomic expression of the LDL receptor, we evaluated their individual and concerted effects on LDL receptor messenger RNA (mRNA) accumulation, stability, and gene promoter activity in first passage monolayer (serum-free) cultures of porcine granulosa cells. Ribonuclease protection assays revealed that LDL receptor mRNA accumulation was increased by human recombinant IGF-I (100 ng/ml), FSH (25 ng/ml NIDDK oFSH-20), or their combination by 2.2-, 2.6-, and 4.6-fold, respectively ($P < 0.01$). Hormonally stimulated LDL receptor mRNA accumulation was suppressed by 54-75% by the concurrent addition of LDL substrate (50 microg/ml). The combination of FSH and IGF-I significantly prolonged the message half-life, even in the presence of LDL. Using a combination of rapid **amplification** of cDNA 5'-ends, PCR with adapter-ligated genomic DNA, Southern hybridization, and DNA sequencing, we isolated 1076 bp of the porcine LDL receptor gene upstream of the coding region. In transient transfection assays, with a pLDLR1076/luciferase plasmid construct, FSH, FSH plus IGF-I, or 8-bromo-cAMP (1 mM) treatment (but not IGF-I alone) increased luciferase **reporter** gene activity by 10- to 23-fold in porcine granulosa cells. Over time in serum-free culture, the basal activity of the LDL receptor gene promoter increased and eventually surpassed

hormone-stimulated effects, but was suppressed by LDL substrate (by 75%) at 24 h. The foregoing stimulatory hormone effects and sterol repression were localized to a 116-bp region in the porcine promoter between -255 and
-139 upstream of the translational start site. We conclude that the combination of FSH and IGF-I can induce accumulation of LDL receptor mRNA in cultured granulosa cells even in the presence of sterol negative feedback and can do so mechanistically by a combination of promoter activation and increased mRNA stability.

L15 ANSWER 4 OF 21 MEDLINE
ACCESSION NUMBER: 1999097055 MEDLINE
DOCUMENT NUMBER: 99097055
TITLE: Interaction of osmotic stress, temperature, and abscisic acid in the regulation of gene expression in Arabidopsis.
AUTHOR: Xiong L; Ishitani M; Zhu J K
CORPORATE SOURCE: Department of Plant Sciences, University of Arizona, Tucson, Arizona 85721, USA.
SOURCE: PLANT PHYSIOLOGY, (1999 Jan) 119 (1) 205-12.
Journal code: P98. ISSN: 0032-0889.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199907
ENTRY WEEK: 19990702
AB The impact of simultaneous environmental stresses on plants and how they respond to combined stresses compared with single stresses is largely unclear. By using a transgene (RD29A-LUC) consisting of the firefly luciferase coding sequence (LUC) driven by the stress-responsive RD29A promoter, we investigated the interactive effects of temperature, osmotic stress, and the phytohormone abscisic acid (ABA) in the regulation of gene
expression in Arabidopsis seedlings. Results indicated that both positive and negative interactions exist among the studied stress factors in regulating gene expression. At a normal growth temperature (22 degrees C),
osmotic stress and ABA act **synergistically** to induce the transgene expression. Low temperature inhibits the response to osmotic stress or to combined treatment of osmotic stress and ABA, whereas low temperature and ABA treatments are additive in inducing transgene expression. Although high temperature alone does not activate the transgene, it significantly **amplifies** the effects of ABA and osmotic stress. The effect of multiple stresses in the regulation of RD29A-LUC expression in signal transduction mutants was also studied. The results are discussed in the context of cold and osmotic stress signal transduction pathways.

L15 ANSWER 5 OF 21 MEDLINE
ACCESSION NUMBER: 1999097021 MEDLINE
DOCUMENT NUMBER: 99097021
TITLE: **Amplification** of gene expression using both 5'- and 3'-untranslated regions of GLUT1 glucose transporter mRNA.
AUTHOR: Boado R J; Pardridge W M
CORPORATE SOURCE: Department of Medicine and Brain Research Institute, UCLA School of Medicine, Los Angeles, CA 90095, USA..
rboado@med1.medsch.ucla.edu
CONTRACT NUMBER: NS-25554 (NINDS)
SOURCE: BRAIN RESEARCH. MOLECULAR BRAIN RESEARCH, (1999 Jan 8) 63 (2) 371-4.
Journal code: MBR. ISSN: 0169-328X.
PUB. COUNTRY: Netherlands
Journal; Article;. (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199904
ENTRY WEEK: 19990404
AB Cis-regulatory elements located at either the 5'- or 3'-untranslated

region (UTR) of the GLUT1 glucose transporter mRNA increase the expression of luciferase **reporter** genes. The aim of the present study was to investigate the possible **cooperative** effects of 5'- and 3'-UTRs of the GLUT1 mRNA on the expression of a luciferase **reporter** gene in cultured brain endothelial cells. Luciferase **reporter** genes containing control elements in nucleotides (nt) 1-171 of GLUT1 5'-UTR, or nt 2100-2300 of GLUT1 3'-UTR produced a 10- and 6-fold increase in the expression of the luciferase **reporter** gene compared to the control vector containing no GLUT1 regulatory sequences, respectively. The insertion of both GLUT1 mRNA cis-regulatory elements increased 59-fold the activity of luciferase compared to controls. Data presented here demonstrate that cis-regulatory elements located at both the 5'- and 3'-UTR of GLUT1 mRNA increase expression of a **reporter** gene in an independent manner. Copyright 1999 Elsevier Science B.V.

L15 ANSWER 6 OF 21 MEDLINE
ACCESSION NUMBER: 1998427285 MEDLINE
DOCUMENT NUMBER: 98427285
TITLE: Potentiation of nitric oxide synthase expression by superoxide in interleukin 1 beta-stimulated rat mesangial cells.
AUTHOR: Beck K F; Eberhardt W; Walpen S; Apel M; Pfeilschifter J
CORPORATE SOURCE: Zentrum der Pharmakologie, Klinikum der Johann Wolfgang Goethe-Universitat, Frankfurt am Main, Germany.
SOURCE: FEBS LETTERS, (1998 Sep 11) 435 (1) 35-8.
Journal code: EUH. ISSN: 0014-5793.
PUB. COUNTRY: Netherlands
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals; Cancer Journals
ENTRY MONTH: 199812
ENTRY WEEK: 19981202

AB Exposure of mesangial cells to superoxide, generated by the hypoxanthine/xanthine oxidase system or by the redox cycler 2,3-dimethoxy-1,4-naphthoquinone caused a concentration-dependent **amplification** of interleukin (IL)-1beta-stimulated nitrite production. The effect of superoxide was accompanied by an increase in inducible nitric oxide synthase (iNOS) protein and iNOS mRNA levels. Incubation of mesangial cells with superoxide alone did not induce iNOS expression. To elucidate whether the increase of iNOS expression is due to transcriptional upregulation we fused a 4.5-kb genomic iNOS fragment that contains the transcriptional start site of the rat iNOS gene to a luciferase **reporter** gene. In transient transfection studies, superoxide caused a 10-fold augmentation of iNOS promoter activity in IL-1beta-challenged mesangial cells. Our data identify superoxide as a co-stimulatory factor **amplifying** cytokine-induced iNOS gene expression and subsequent nitric oxide (NO) synthesis.

L15 ANSWER 7 OF 21 MEDLINE
ACCESSION NUMBER: 1998075070 MEDLINE
DOCUMENT NUMBER: 98075070
TITLE: Overexpression of adenovirus-encoded transgenes from the cytomegalovirus immediate early promoter in irradiated tumor cells.
AUTHOR: Tang D C; Jennelle R S; Shi Z; Garver R I Jr; Carbone D P; Loya F; Chang C H; Curiel D T
CORPORATE SOURCE: Department of Medicine, University of Alabama at Birmingham, 35294, USA.
SOURCE: HUMAN GENE THERAPY, (1997 Nov 20) 8 (17) 2117-24.
Journal code: A12. ISSN: 1043-0342.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199804
ENTRY WEEK: 19980404

AB Efficient expression of therapeutic genes in irradiated tumor cells would facilitate the conversion of a malignant tumor nodule to a cancer vaccine in situ. We reported previously that transgene expression from an adenoviral vector could be markedly enhanced by treating transduced tumor cells with butyrate. In this study, we demonstrated that a similar butyrate effect could be achieved in irradiated tumor cells. In addition, irradiating cells at doses of 2-40 Gy prior to transduction could also **amplify** recombinant adenoviral transgene products in a cell-type-specific manner. This suggests that adenovirus-mediated gene therapy, radiation therapy, and butyrate-mediated cancer therapy may potentially be formulated into one **synergistic** protocol for cancer treatment.

L15 ANSWER 8 OF 21 MEDLINE
ACCESSION NUMBER: 97156647 MEDLINE
DOCUMENT NUMBER: 97156647
TITLE: Interactions of muscarinic receptors with the heterotrimeric G proteins Gq and G12: transduction of proliferative signals.
AUTHOR: Burstein E S; Brauner-Osborne H; Spalding T A; Conklin B R;
Brann M R
CORPORATE SOURCE: Department of Psychiatry, University of Vermont, Burlington, USA.
CONTRACT NUMBER: R01 GM52737 (NIGMS)
F32NS09436 (NINDS)
SOURCE: JOURNAL OF NEUROCHEMISTRY, (1997 Feb) 68 (2) 525-33.
Journal code: JAV. ISSN: 0022-3042.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199705

AB The proliferative and transforming properties of m2 and m5 muscarinic acetylcholine receptors and a series of wild-type, chimeric, and mutant G proteins were measured alone or in combination in NIH 3T3 cells to determine which G proteins mediate these signals and to what extent these signals can be influenced by changing the stoichiometry of receptors and G proteins. Responses were measured using the focus-forming assay and a novel assay called R-SAT (Receptor Selection and **Amplification** Technology) in which proliferative responses are monitored using a **reporter** gene. Individually, GTPase-deficient mutants (*) of G alpha q and G alpha 12, wild-type G alpha q, and m5 were active in R-SAT. G alpha 12* and m5 also induced focus formation. m2 was inactive in both assays. The ability of m5 to induce foci was significantly reduced by coexpression of G alpha q*. **Synergistic** effects of receptor/ G protein combinations were not observed in focus-forming assays but were readily detected by R-SAT. Coexpression of G alpha q with m5 induced constitutive activity in R-SAT and increased the potency of agonists at m5 by 90-fold. G alpha q also evoked agonist-dependent responses from m2 but not constitutive activity. Agonist potency was increased 10-fold at m2 and decreased 15-fold at m5 when these receptors were coexpressed with G alpha qi5, a chimeric G protein containing the five C-terminal residues of G alpha i2, compared with coexpression with G alpha q. Both G alpha q and G alpha qi5 had biphasic effects on the proliferative responses to m5 and m2, respectively, inhibiting responses at high agonist concentrations. Coexpression of G alpha 12 or G alpha 12i5 had no effect on the concentration-response relationships of m5, but both elicited weak responses from m2. We conclude that although G alpha 12 is a more potent oncogene, G alpha q transduces m5-driven cellular responses. The demonstrations that proliferative responses can be elicited from a nonmitogenic receptor by altering the type and concentration of available G proteins and that constitutive responses can be induced by G proteins imply that both the magnitude and type of receptor-initiated signal can be

regulated at the level of G proteins in vivo.

L15 ANSWER 9 OF 21 MEDLINE
ACCESSION NUMBER: 96432327 MEDLINE
DOCUMENT NUMBER: 96432327
TITLE: Transcriptional regulation of sertoli cell differentiation by follicle-stimulating hormone at the level of the c-fos and transferrin promoters.
AUTHOR: Chaudhary J; Whaley P D; Cupp A; Skinner M K
CORPORATE SOURCE: Reproductive Endocrinology Center, University of California, San Francisco 94143-0556, USA.
CONTRACT NUMBER: HD 20583 (NICHD)
SOURCE: BIOLOGY OF REPRODUCTION, (1996 Mar) 54 (3) 692-9. Journal code: A3W. ISSN: 0006-3363.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199702

AB One of the primary endocrine hormones that influence the onset of Sertoli cell differentiation at puberty and help maintain differentiation in the adult testis is FSH. FSH can modulate the majority of Sertoli cell differentiated functions, including stimulation of the iron-binding protein transferrin. Previous studies have shown that FSH alters the levels of cAMP and the immediate early gene c-fos. The current study was designed to investigate the transcriptional regulation of Sertoli cell differentiation by examining the actions of FSH on the promoter of the immediate early gene c-fos and the promoter of the downstream differentiated function gene transferrin. The regulation of c-fos by FSH was investigated with various chloramphenicol acetyltransferase (CAT) constructs containing segments of the c-fos promoter, such as the serum response element (SRE), cAMP response element (CRE), and AP1/phorbol ester/TPA response element (TRE), that were transfected into cultured Sertoli cells. Observations indicate that FSH can stimulate all three response elements, as well as a whole c-fos promoter construct. Interestingly, FSH was found to have a more dramatic effect on the SRE-CAT than a cAMP analog, suggesting a difference in the actions of the two agents. Gel mobility shift assays were performed to confirm the **reporter** gene results. Nuclear extracts of FSH-stimulated Sertoli cells caused a labeled AP1 oligonucleotide to form a DNA/protein complex (i.e., gel shift), indicating activation of the c-fos gene and binding of the c-fos/jun complex. Nuclear extracts from both FSH- and cAMP-stimulated Sertoli cells promoted similar gel shifts with SRE and CRE oligonucleotides. This observation supports the **reporter** gene data in indicating that FSH can influence both the SRE and CRE. A gel mobility shift assay was also performed with an oligonucleotide containing the 5'-flanking ETS domain of the SRE (ETS-SRE) that allows the formation of a ternary complex. FSH-stimulated Sertoli cell nuclear extracts were found to promote a unique ETS-SRE gel shift not present in cAMP-stimulated cells. The observations imply that FSH actions on the SRE are in part distinct from the actions of cAMP. Transferrin gene expression was examined to study the downstream regulation of Sertoli cell differentiation. CAT constructs containing deletion mutants of a 3-kb mouse transferrin promoter were used. When transfected into Sertoli cells, the 581-bp transferrin minimal promoter, previously shown to contain a CRE, had a significant response to cAMP and FSH. The 1.6-, 2.6-, and 3-kg transferrin promoter constructs also responded to FSH and cAMP to the same extent as, or to a lesser extent than, the 581-bp minimal promoter. Interestingly, the actions of FSH on the 581-bp minimal transferrin promoter were more dramatic than those of cAMP. The importance of FSH-induced c-fos in the regulation of transferrin expression was demonstrated in the current study when a c-fos antisense oligonucleotide was found to partially inhibit (50%) the ability of FSH to induce the

expression of a transferrin promoter (CAT) construct. Therefore, FSH appears to act through multiple transcriptional activation pathways. The first involves cAMP and the CRE at both early-event genes (e.g., c-fos) and downstream genes (e.g., transferrin). It is likely that other pathways involve alternate signal transduction events (e.g., calcium mobilization) and promoter response elements (e.g., SRE). These multiple pathways may act in a compensatory manner to assure the ability of FSH to influence Sertoli cell differentiation and/or in a **synergistic** manner to **amplify** FSH actions.

L15 ANSWER 10 OF 21 MEDLINE
ACCESSION NUMBER: 96407841 MEDLINE
DOCUMENT NUMBER: 96407841
TITLE: Identification of cis-elements regulating the expression of an Arabidopsis histone H4 gene.
AUTHOR: Chaubet N; Flenet M; Clement B; Brignon P; Gigot C
CORPORATE SOURCE: Institut de Biologie Moleculaire des Plantes du CNRS, Universite Louis Pasteur, Strasbourg, France.
SOURCE: PLANT JOURNAL, (1996 Sep) 10 (3) 425-35.
Journal code: BRU. ISSN: 0960-7412.
PUB. COUNTRY: ENGLAND: United Kingdom
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199704
ENTRY WEEK: 19970402
AB Protein-DNA interactions in the proximal region of an Arabidopsis H4 histone gene promoter were analyzed by DMS in vivo footprinting combined with LMPCR **amplification**. Interactions were identified over six particular sequence motifs, five of which were previously shown to bind proteins in maize histone H3 and H4 promoters and are commonly found in the corresponding regions of other plant histone gene promoters. These motifs are located within a 126 bp fragment which was previously shown to confer preferential expression in meristems of transgenic plants. The contribution of each cis-element to the overall expression level and specificity was investigated by testing individual or combined mutations in transgenic Arabidopsis plants. All five motifs behaved as positive cis-elements of unequal strength. The GCCAAT-like sequence GCCACT behaved as a strong positive cis-element but had no influence on the specificity. In contrast, the nonamer AGATCGACG and to a lesser extent the closely linked hexamer CCGTCG proved to be essential for meristem-specific expression. Involvement of the highly conserved histone-specific octamer CGCGGATC in specific expression was revealed at some stages of meristem development. Importance of these three cis-elements, nonamer, hexamer, and octamer, was further confirmed by the fact that combining mutations of two of them either abolished the promoter activity or completely modified the promoter specificity. Mutation of the fifth cis-element, a degenerate copy of the octamer, little perturbed the promoter function. However disruption of both octamers had a dramatic negative effect, thus suggesting that the two copies **cooperate** to achieve maximal function in the wild-type promoter, possibly by mobilizing the proliferation-specific factors binding to the nonamer and CCGTCG cis-elements.

L15 ANSWER 11 OF 21 MEDLINE
ACCESSION NUMBER: 95237963 MEDLINE
DOCUMENT NUMBER: 95237963
TITLE: Fra-1, a Fos gene family member that activates atrial natriuretic peptide gene transcription.
AUTHOR: Kovacic-Milivojevic B; Gardner D G
CORPORATE SOURCE: Metabolic Research Unit, University of California at San Francisco 94143, USA.
CONTRACT NUMBER: HL-35753 (NHLBI)
SOURCE: HYPERTENSION, (1995 Apr) 25 (4 Pt 2) 679-82.

Journal code: GK7. ISSN: 0194-911X.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199507

AB Previous studies suggested that individual components of the activator protein 1 (AP-1) complex behave in a highly idiosyncratic fashion at the level of the human atrial natriuretic peptide (ANP) gene promoter. ANP gene transcription is activated by c-jun and is generally suppressed by c-fos. In the present study, fra-1, a close relative of the c-fos gene product in terms of its structure and functional activity, behaved like fos in cardiac atriocytes, effecting an approximately 50% reduction in c-jun-activatable expression of a human ANP chloramphenicol acetyltransferase (CAT) **reporter**. In cardiac ventriculocytes, however, fra-1 effected a **synergistic amplification** of the c-jun response (a 2.5-fold increase over c-jun alone). In atrial cells, fos-like proteins were not uniformly inhibitory in that a carboxy terminal deletion mutant of c-fos activated a human ANP-CAT **reporter** in the atriocyte cultures. Finally, using a series of domain-swap mutations in the fos/fra structural sequences, we showed that sequences at both the amino and the carboxy termini are required to realize the full fra-1-dependent stimulatory effect as well as the c-fos-dependent inhibition of ANP gene transcription. These findings suggest considerable heterogeneity in the response of the ANP promoter to different components of the AP-1 complex. Such heterogeneity may serve to broaden the range of biological responses available to this promoter as the cardiac cell attempts to adapt to perturbations in the extracellular environment.

L15 ANSWER 12 OF 21 MEDLINE
ACCESSION NUMBER: 93373853 MEDLINE
DOCUMENT NUMBER: 93373853
TITLE: Differential regulation of human progesterone receptor A and B form-mediated trans-activation by phosphorylation.
AUTHOR: Kazmi S M; Visconti V; Plante R K; Ishaque A; Lau C
CORPORATE SOURCE: Biological Research Department, R. W. Johnson Pharmaceutical Research Institute, Don Mills, Ontario, Canada.
SOURCE: ENDOCRINOLOGY, (1993 Sep) 133 (3) 1230-8.
Journal code: EGZ. ISSN: 0013-7227.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Abridged Index Medicus Journals; Priority Journals; Cancer Journals
ENTRY MONTH: 199312

AB Hormone-dependent phosphorylation of progesterone receptors (PRs) plays a functional role in their transcriptional activity. However, hormone-independent phosphorylation has also been shown to modulate the chicken PR-mediated trans-activation in the presence of phosphorylating agents. The present study was designed to investigate the effects of protein kinase A- and protein kinase C-mediated signal transduction pathways on the regulation of the activity of the two forms of human PR (hPRA and hPRB). Similar to chicken PR, hPR was activated by 8-bromo-cAMP (8-Br-cAMP) in the absence of ligand, whereas 8-Br-cAMP **synergized** with the progestin agonist R5020 to **amplify** hPRA- and hPRB-mediated **reporter** activity. Interestingly, the effect of 8-Br-cAMP was much more pronounced on hPRA-induced trans-activation than on hPRB. This differential regulation by 8-Br-cAMP could also be mimicked by okadaic acid. Both mouse mammary tumor virus-thymidine kinase-chloramphenicol acetyl transferase and progesterone response element-thymidine kinase-chloramphenicol acetyl transferase showed a similar response to 8-Br-cAMP in the presence of R5020. Protein kinase C, on the other hand, did not discriminate between hPRA- and hPRB-mediated trans-activation. Unlike 8-Br-cAMP, phorbol 12-myristate 13-acetate did not cause marked ligand-independent trans-activation through either of the two receptor forms. RU486, an antagonist of progestin, preferentially

blocked R5020-induced trans-activation compared to R5020 + 8-Br-cAMP **synergism**. As expected, H-89, a specific inhibitor of protein kinase A was more effective in inhibiting ligand-independent activity. Western analysis of transfected receptors suggested that 8-Br-cAMP and 8-Br-cAMP + R5020 but not R5020 alone down-regulated the level of hPRB in COS-1 cells. Only marginal modulation of hPRA levels was observed with R5020 treatment in the presence and absence of 8-Br-cAMP. These data suggest that R5020 and 8-Br-cAMP mediate PR-dependent transactivation through distinct pathways, and that phosphorylation can differentially regulate the activity of hPRA and hPRB forms, an observation which may be important for selective target gene activation in vivo by progestins.

L15 ANSWER 13 OF 21 MEDLINE
ACCESSION NUMBER: 93324001 MEDLINE
DOCUMENT NUMBER: 93324001
TITLE: **Amplification** of calcium-induced gene transcription by nitric oxide in neuronal cells [published erratum appears in Nature 1993 Sep 30;365(6445):468].
AUTHOR: Peunova N; Enikolopov G
CORPORATE SOURCE: Cold Spring Harbor Laboratory, New York 11724.
SOURCE: NATURE, (1993 Jul 29) 364 (6436) 450-3.
Journal code: NSC. ISSN: 0028-0836.
PUB. COUNTRY: ENGLAND: United Kingdom
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Cancer Journals; Priority Journals
ENTRY MONTH: 199310
AB Nitric oxide (NO) is a short-lived, highly reactive gas, which has been identified as a mediator in vasodilation, an active agent in macrophage cytotoxicity and neurotoxicity, and a neuro-transmitter in the central and peripheral nervous systems. Production of NO by neurons is critical for facilitated synaptic transmission in models of synaptic plasticity such as long-term potentiation and long-term depression, suggesting a role for NO as a retrograde messenger that could complete a hypothetical feedback loop by strengthening the connection between postsynaptic and presynaptic cells. We report here that although alone NO has no evident effect on transcription, it can act as an **amplifier** of calcium signals in neuronal cells. NO and Ca2+ action have to coincide in time for **amplification** to occur. Experiments with a series of simplified **reporter** genes in combination with specific recombinant protein kinase inhibitors suggest that induction of gene activity following NO-**amplified** calcium action involves protein kinase A-dependent activation of the transcription factor CREB.

L15 ANSWER 14 OF 21 MEDLINE
ACCESSION NUMBER: 89096932 MEDLINE
DOCUMENT NUMBER: 89096932
TITLE: trans activation of type 1 interferon promoters by simian virus 40 T antigen.
AUTHOR: Hiscott J; Wong A; Alper D; Xanthoudakis S
CORPORATE SOURCE: Lady Davis Institute for Medical Research, Sir Mortimer B. Davis--Jewish General Hospital, Montreal, Quebec, Canada.
SOURCE: MOLECULAR AND CELLULAR BIOLOGY, (1988 Aug) 8 (8) 3397-405.
Journal code: NGY. ISSN: 0270-7306.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 198904
AB A human transient expression system was used to measure the influence of simian virus 40 T antigen and adenovirus E1a proteins on the activation of alpha interferon subtype 1 (IFN-alpha 1) and IFN-beta promoters linked to the **reporter** chloramphenicol acetyltransferase gene. Large T-antigen production, **amplified** by expression plasmid replication in transfected 293 cells, was able to trans activate the

IFN-beta promoter 5-[redacted]10-fold, increasing both the constitutive and Sendai virus-induced [redacted]vels of expression. Surprising[redacted] the previously quiescent transfected IFN-alpha 1 promoter in T-antigen-expressing cells displayed a level of inducibility similar to IFN-beta. The endogenous IFN-alpha 1 gene was also inducible to a limited extent in cells expressing T antigen. A truncated IFN-beta promoter deleted to position -37 relative to the CAP site was neither inducible nor trans activated by T antigen, suggesting that sequences required for efficient induction were also needed for trans activation. Since 293 cells express adenoviral E1a proteins, experiments were also performed in HeLa cells to assess the relative contribution of T antigen and E1a proteins to IFN trans activation. In HeLa cells, T-antigen coexpression increased the constitutive level of IFN-beta and IFN-alpha 1 promoter activity without augmenting relative inducibility. Coexpression of T antigen and E1a proteins did not have a **cooperative** effect on type 1 IFN expression.

L15 ANSWER 15 OF 21 BIOSIS COPYRIGHT 2000 BIOSIS
ACCESSION NUMBER: 2000:8254 BIOSIS
DOCUMENT NUMBER: PREV200000008254
TITLE: Productivity enhancement of recombinant protein in CHO cells via specific promoter activation by oncogenes.
AUTHOR(S): Katakura, Yoshinori (1); Seto, Perry; Miura, Takumi; Ohashi, Hideya; Teruya, Kiichiro; Shirahata, Sanetaka
CORPORATE SOURCE: (1) Graduate School of Genetic Resources Technology, Kyushu University, Hakozaki 6-10-1, Higashi-ku, Fukuoka, 812-8581 Japan
SOURCE: Cytotechnology, (1999) Vol. 31, No. 1-2, pp. 103-109. ISSN: 0920-9069.
DOCUMENT TYPE: Article
LANGUAGE: English
SUMMARY LANGUAGE: English
AB To construct a recombinant protein highly producing cell lines, we have previously developed the Oncogene Activated Production (OAP) system by using BHK-21 cells. Here we verified the availability of the OAP system in CHO cells. We firstly generated 'primed' ras **amplified** CHO cells, ras clone I, by introducing human c-Ha-ras oncogene into CHO cells. This ras clone I enables quick and easy establishment of recombinant protein hyper producing cell lines by introduction **reporter** gene of interest. Then we generated I13 by introducing human interleukin 6 (hIL-6) gene as a **reporter** gene, which showed enhanced productivity rate as compared to A7 established by conventional method. Furthermore, we found that hIL-6 production level of I13 was slightly improved by raising the CO2 concentration from 5 to 8% possibly because of the enhanced growth rate. We further introduced the E1A oncogene, which has been shown to have a **synergistic** effect on the recombinant protein production of the ras-**amplif** ied BHK-21 cells, then evaluated the productivity. When culture in 5% CO2 condition, only the slight effect can be seen. However when cultured in 8% CO2 condition, not only cell number, but also productivity increased significantly, resulted in great augmentation of hIL-6 production, maximum production being 88.6 mug/ml/3 days. This study demonstrates that recombinant protein production level reached commercially desirable level by utilizing our OAP system in CHO cells and optimizing the culture condition.

L15 ANSWER 16 OF 21 BIOSIS COPYRIGHT 2000 BIOSIS
ACCESSION NUMBER: 1999:317311 BIOSIS
DOCUMENT NUMBER: PREV199900317311
TITLE: Complete cDNA cloning, genomic organization, chromosomal assignment, functional characterization of the promoter, and expression of the murine bamacan gene.
AUTHOR(S): Ghiselli, Giancarlo; Siracusa, Linda D.; Iozzo, Renato V. (1)

CORPORATE SOURCE: (1) De[redacted] of Pathology, Anatomy, and Ce[redacted] Biology, JAH, Thomas [redacted]efferson University, 1020 Locus[redacted]t., Rm. 249, Philadelphia, PA, 19107 USA
SOURCE: Journal of Biological Chemistry, (June 11, 1999) Vol. 274, No. 24, pp. 17384-17393.
ISSN: 0021-9258.
DOCUMENT TYPE: Article
LANGUAGE: English
SUMMARY LANGUAGE: English

AB Bamacan is a chondroitin sulfate proteoglycan that abounds in basement membranes. To gain insights into the bamacan gene regulation and transcriptional control, we examined the genomic organization and identified the promoter region of the mouse bamacan gene. Secondary structure analysis of the protein reveals a sequential organization of three globular regions interconnected by two alpha-helix coiled-coils. The N- and C-terminal ends carry a P-loop and a DA box motif that can act **cooperatively** to bind ATP. These features as well as the high sequence homology with members of the SMC (structural maintenance of chromosome) protein family led us to conclude that bamacan is a member of this protein family. The gene comprises 31 exons and is driven by a promoter that is highly enriched in GC sequences and lacks TATA and CAAT boxes. The promoter is highly functional in transient cell transfection assays, and step-wise 5' deletions identify a strong enhancer element between -659 and -481 base pairs that includes Jun/Fos proto-oncogene-binding elements. Using back-crossing experiments we mapped the Bam gene to distal chromosome 19, a locus syntenic to human chromosome 10q25. Bamacan is differentially expressed in mouse tissues with the highest levels in testes and brain. Notably, bamacan mRNA levels are low in normal cells and markedly reduced during quiescence but are highly increased when cells resume growth upon serum stimulation. In contrast, in all transformed cells tested, bamacan is constitutively overexpressed, and its levels do not change with cell cycle progression. These results suggest that bamacan is involved in the control of cell growth and transformation.

L15 ANSWER 17 OF 21 BIOSIS COPYRIGHT 2000 BIOSIS
ACCESSION NUMBER: 1999:154381 BIOSIS
DOCUMENT NUMBER: PREV199900154381
TITLE: Characterization of a novel calcium response element in the glucagon gene.
AUTHOR(S): Fuerstenau, Ursel; Schwaninger, Markus; Blume, Roland; Jendrusch, Eva-Maria; Knepel, Willhart (1)
CORPORATE SOURCE: (1) Dep. Mol. Pharmacol., Univ. Goettingen, Robert-Koch-Str. 40, D-37070 Goettingen Germany
SOURCE: Journal of Biological Chemistry, (Feb. 26, 1999) Vol. 274, No. 9, pp. 5851-5860.
ISSN: 0021-9258.
DOCUMENT TYPE: Article
LANGUAGE: English

AB To maintain blood glucose levels within narrow limits, the synthesis and secretion of pancreatic islet hormones is controlled by a variety of extracellular signals. Depolarization-induced calcium influx into islet cells has been shown to stimulate glucagon gene transcription through the transcription factor cAMP response element-binding protein that binds to the glucagon cAMP response element. By transient transfection of glucagon-**reporter** fusion genes into islet cell lines, this study identified a second calcium response element in the glucagon gene (G2 element, from -165 to - 200). Membrane depolarization was found to induce the binding of a nuclear complex with NFATp-like immunoreactivity to the G2 element. Consistent with nuclear translocation, a comigrating complex was found in cytosolic extracts of unstimulated cells, and the induction of nuclear

protein binding was b[redacted]ked by inhibition of calcineur[redacted] phosphatase activity by FK506. A [redacted]tational analysis of G2 functio[redacted]nd nuclear protein binding as well as the effect of FK506 indicate that calcium responsiveness is conferred to the G2 element by NFATP functionally interacting with HNF-3beta binding to a closely associated site. Transcription factors of the NFAT family are known to **cooperate** with AP-1 proteins in T cells for calcium-dependent activation of cytokine genes. This study shows a novel pairing of NFATP with the cell lineage-specific transcription factor HNP-3beta in islet cells to form a novel calcium response element in the glucagon gene.

L15 ANSWER 18 OF 21 BIOSIS COPYRIGHT 2000 BIOSIS
ACCESSION NUMBER: 1999:110823 BIOSIS
DOCUMENT NUMBER: PREV199900110823
TITLE: Characterization of the human endothelial nitric-oxide synthase promoter.
AUTHOR(S): Karantzoulis-Fegaras, Fotula; Antoniou, Hariclia; Lai, Sheue-Lim M.; Kulkarni, Girish; D'Abreo, Cheryl; Wong, Gordon K. T.; Miller, Tricia L.; Chan, Yvonne; Atkins, Judith; Wang, Yang; Marsden, Philip A. (1)
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SOURCE: Journal of Biological Chemistry, (Jan. 29, 1999) Vol. 274, No. 5, pp. 3076-3093.
ISSN: 0021-9258.
DOCUMENT TYPE: Article
LANGUAGE: English
AB Understanding transcription initiation of the endothelial nitric-oxide synthase (eNOS) gene appears pivotal to gaining a comprehensive view of NO biology in the blood vessel wall. The present study therefore focused upon a detailed dissection of the functionally important cis-DNA elements and the multiprotein complexes implicated in the **cooperative** control of constitutive expression of the human eNOS gene in vascular endothelium.
Two tightly clustered cis-regulatory regions were identified in the proximal enhancer of the TATA-less eNOS promoter using deletion analysis and linker-scanning mutagenesis: positive regulatory domains I (-104/-95 relative to transcription initiation) and II (-144/-115). Analysis of trans-factor binding and functional expression studies revealed a surprising degree of **cooperativity** and complexity. The nucleoprotein complexes that form upon these regions in endothelial cells contained Ets family members, Sp1, variants of Sp3, MAZ, and YY1. Functional domain studies in Drosophila Schneider cells and endothelial cells revealed examples of positive and negative protein-protein **cooperativity** involving Sp1, variants of Sp3, Ets-1, Elf-1, and MAZ. Therefore, multiprotein complexes are formed on the activator recognition sites within this 50-base pair region of the human eNOS promoter in vascular endothelium.

L15 ANSWER 19 OF 21 BIOSIS COPYRIGHT 2000 BIOSIS
ACCESSION NUMBER: 1999:31694 BIOSIS
DOCUMENT NUMBER: PREV199900031694
TITLE: Two adjacent protein binding motifs in the cbh2 (cellobiohydrolase II-encoding) promoter of the fungus Hypocrea jecorina (Trichoderma reesei) **cooperate** in the induction by cellulose.
AUTHOR(S): Zeilinger, Susanne (1); Mach, Robert L.; Kubicek, Christian P.
CORPORATE SOURCE: (1) Abteilung Mikrobielle Biochem., Inst. Biochem. Technol. Mikrobiol., Tech. Univ. Wien, Getreidemarkt 9/1725, A-1060 Wien Austria
SOURCE: Journal of Biological Chemistry, (Dec. 18, 1998) Vol. 273, No. 51, pp. 34463-34471.
ISSN: 0021-9258.

DOCUMENT TYPE: Articl
LANGUAGE: Englis

AB The cellulase system of the filamentous fungus Hypocrea jecorina (Trichoderma reesei) consists of several cellobiohydrolases, endoglucanases, and beta-glucosidases, encoded by separate genes, which are coordinately expressed in the presence of cellulose or the disaccharide sophorose. Using cell-free extracts from sophorose-induced and noninduced mycelia and various fragments of the cbh2 promoter of H. jecorina in electrophoretic mobility shift assay (EMSA) analysis and performing in vitro and in vivo footprinting analysis, we detected the nucleotide sequence 5'-ATTGGGTAATA-3' (consequently named cbh2-activating element (CAE)) to bind a protein complex with different migration in EMSA of induced and noninduced cell-free extracts. EMSA analysis, employing oligonucleotide fragments containing specifically mutated versions of CAE, revealed that protein binding requires the presence of an intact copy of either one of two adjacent motifs: a CCAAT (=ATTGG) box on the template strand and a GTAATA box on the coding strand, whereas a simultaneous mutation in both completely abolished binding. H. jecorina transformants, containing correspondingly mutated versions of the cbh2 promoter fused to the Escherichia coli hph gene as a **reporter**, expressed hph in a manner paralleling the efficacy of CAE-protein complex formation in EMSA, suggesting that the presence of either of both motifs is required for induction of cbh2 gene transcription. Antibody supershift experiments with anti-HapC antiserum as well as EMSA competition experiments with CCAAT binding promoter fragments of the Aspergillus nidulans amdS promoter suggest that the H. jecorina CCAAT box binding complex contains a homologue of HapC. The nature of the adjacent, GTAATA-binding protein(s) and its **cooperation** with the HapC homologue in cbh2 gene induction is discussed.

L15 ANSWER 20 OF 21 BIOSIS COPYRIGHT 2000 BIOSIS
ACCESSION NUMBER: 1998:491155 BIOSIS
DOCUMENT NUMBER: PREV199800491155
TITLE: **Synergistic** activation of the N-methyl-D-aspartate receptor subunit 1 promoter by myocyte enhancer factor 2C and Sp1.
AUTHOR(S): Krainc, Dimitri; Bai, Guang; Okamoto, Shu-Ichi; Carles, Maria; Kusiak, John W.; Brent, Roger N.; Lipton, Stuart A. (1)
CORPORATE SOURCE: (1) CNS Res. Inst., Brigham and Women's Hosp., 221 Longwood Ave., LMRC First Floor, Boston, MA 02115 USA
SOURCE: Journal of Biological Chemistry, (Oct. 2, 1998) Vol. 273, No. 40, pp. 26218-26224.
ISSN: 0021-9258.
DOCUMENT TYPE: Article
LANGUAGE: English

AB The N-methyl-D-aspartate (NMDA) subtype of glutamate receptor plays important roles in neuronal development, plasticity, and cell death. NMDA receptor subunit 1 (NR1) is an essential subunit of the NMDA receptor and is developmentally expressed in postnatal neurons of the central nervous system. Here we identify on the NR1 promoter a binding site for myocyte enhancer factor 2C (MEF2C), a developmentally expressed neuron/muscle transcription factor found in cerebrocortical neurons, and study its regulation of the NR1 gene. Co-expression of MEF2C and Sp1 cDNAs in primary neurons or cell lines **synergistically** activates the NR1 promoter. Disruption of the MEF2 site or the MEF2C DNA binding domain moderately reduces this **synergism**. Mutation of the Sp1 sites or the activation domains of Sp1 protein strongly reduces the **synergism**. Results of yeast two-hybrid and co-immunoprecipitation experiments reveal a physical interaction between MEF2C and Sp1 proteins. The MEF2C DNA binding domain is sufficient for this interaction. Dominant-negative MEF2C interferes with expression of NR1 mRNA in neuronally differentiated P19 cells. Growth factors, including epidermal growth factor and basic fibroblast growth factor, can up-regulate NR1 promoter activity in stably transfected PC12 cells, even in the absence of

the MEF2 site, but th[redacted]p1 sites are necessary for thi[redacted]rowth factor regulation, suggesting that Sp1 sites may mediate thes[redacted]effects.

L15 ANSWER 21 OF 21 BIOSIS COPYRIGHT 2000 BIOSIS
ACCESSION NUMBER: 1998:404395 BIOSIS
DOCUMENT NUMBER: PREV199800404395
TITLE: The molecular basis of Rieger syndrome: Analysis of PITX2 homeodomain protein activities.
AUTHOR(S): Amendt, Brad A. (1); Sutherland, Lillian B.; Semina, Elena V.; Russo, Andrew F.
CORPORATE SOURCE: (1) Dep. Physiol. Biophysics, Univ. Iowa, Iowa City, IA 52242 USA
SOURCE: Journal of Biological Chemistry, (Aug. 7, 1998) Vol. 273, No. 32, pp. 20066-20072.
ISSN: 0021-9258.
DOCUMENT TYPE: Article
LANGUAGE: English

AB Rieger syndrome is an autosomal-dominant developmental disorder that includes glaucoma and mild craniofacial dysmorphism in humans. Mutations in the Pitx2 homeobox gene have been linked to Rieger syndrome. We have characterized wild type and mutant Pitx2 activities using electrophoretic mobility shift assays, protein binding, and transient transfection assays.

Pitx2 preferentially binds the bicoid homeodomain binding site and transactivates **reporter** genes containing this site. The combination of Pitx2 and another homeodomain protein, Pit-1, yielded a **synergistic** 55-fold activation of the prolactin promoter in transfection assays. Addition of Pit-1 increased Pitx2 binding to the bicoid element in electrophoretic mobility shift assays. Furthermore, we demonstrate specific binding of Pit-1 to Pitx2 in vitro. Thus, wild type Pitx2 DNA binding activity is modulated by protein-protein interactions.

We next studied two Rieger mutants. A threonine to proline mutation (T68P) in the second helix of the homeodomain retained DNA binding activity with the same apparent KD and only about a 2-fold reduction in the Bmax. However, this mutant did not transactivate **reporter** genes containing the bicoid site. The mutant Pitx2 protein binds Pit-1, but there was no detectable **synergism** on the prolactin promoter. A second mutation (L54Q) in a highly conserved residue in helix 1 of the homeodomain yielded an unstable protein. Our results provide insights into the potential mechanisms underlying the developmental defects in Rieger syndrome.

```
=> file medline biosis

COST IN U.S. DOLLARS                                    SINCE FILE      TOTAL
                                                           ENTRY    SESSION
FULL ESTIMATED COST                                         0.15       0.15

FILE 'MEDLINE' ENTERED AT 11:39:28 ON 03 MAY 2000

FILE 'BIOSIS' ENTERED AT 11:39:28 ON 03 MAY 2000
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=> s fus1

L1          155 FUS1

=> s fus1-ste4

L2            0 FUS1-STE4

=> fus1-ste5

FUS1-STE5 IS NOT A RECOGNIZED COMMAND
The previous command name entered was not recognized by the system.
For a list of commands available to you in the current file, enter
"HELP COMMANDS" at an arrow prompt (=>).

=> s fus1-ste5

L3            0 FUS1-STE5

=> fus1 same ste4

FUS1 IS NOT A RECOGNIZED COMMAND
The previous command name entered was not recognized by the system.
For a list of commands available to you in the current file, enter
"HELP COMMANDS" at an arrow prompt (=>).

=> s fus1 same ste4

L4            0 FUS1 SAME STE4

=> fus1-ste?

FUS1-STE? IS NOT A RECOGNIZED COMMAND
The previous command name entered was not recognized by the system.
For a list of commands available to you in the current file, enter
"HELP COMMANDS" at an arrow prompt (=>).

=> s fus1-ste?

L5            0 FUS1-STE?

=> s ste?

L6      1424375 STE?

=> s l6 same l1

MISSING OPERATOR L6 SAME
The search profile that was entered contains terms or
nested terms that are not separated by a logical operator.
```

=> s l6 (s) l1

L7 69 L6 (S) L1

=> dup rem l7

PROCESSING COMPLETED FOR L7
L8 35 DUP REM L7 (34 DUPLICATES REMOVED)

=> d kwic 1-35

L8 ANSWER 1 OF 35 MEDLINE DUPLICATE 1
AB . . . at least 2 self pheromones. The well-investigated pheromone response system of the yeast Saccharomyces cerevisiae was used to link the **FUS1**::lacZ reporter system to the heterologous pheromone receptors from S. commune. To investigate yeast G-protein binding, the unchanged heterologous receptor was compared to constructs carrying an exchange of the 3rd cytoplasmatic loop for the **Ste2** sequence. A better coupling could be achieved with the altered constructs. In order to examine activation by single pheromones, an. . .

L8 ANSWER 2 OF 35 MEDLINE DUPLICATE 2
AB . . . factor or by deletion of the alpha-subunit of the heterotrimeric G-protein. We now show that CLN2 overexpression can also repress **FUS1** induction if the signaling pathway is activated at the level of the beta-subunit of the G-protein (**STE4**) but not when activated at the level of downstream kinases (**STE20** and **STE11**) or at the level of the transcription factor **STE12** . This epistatic analysis indicates that repression of pheromone signaling pathway by Cln2-Cdc28 kinase takes place at a level around **STE20** . In agreement with this, a marked reduction in the electrophoretic mobility of the **Ste20** protein is observed at the time in the cell cycle of maximal expression of CLN2. This mobility change is constitutive. . . absent in cells lacking CLN1 and CLN2. These changes in electrophoretic mobility correlate with repression of pheromone signaling and suggest **Ste20** as a target for repression of signaling by G1 cyclins. Two morphogenic pathways for which **Ste20** is essential, pseudohyphal differentiation and haploid-invasive growth, also require CLN1 and CLN2. Together with the previous observation that Cln1 and Cln2 are required for the function of **Ste20** in cytokinesis, this suggests that Cln1 and Cln2 regulate the biological activity of **Ste20** by promoting morphogenic functions, while inhibiting the mating factor signal transduction function.

L8 ANSWER 3 OF 35 MEDLINE
AB . . . was more active than its lactam-containing diastereomeric homologue WHWLQLK[(S)-gamma-lactam]QP[Nle]Y and about equally active with the [Nle12]-alpha-factor in growth arrest and **FUS1**-lacZ gene induction assays. Both lactam analogues competed with tritiated [Nle12]-alpha-factor for binding to the alpha-factor receptor (**Ste2p**) with the (R)-gamma-lactam-containing peptide having 7-fold higher affinity than the (S)-gamma-lactam-containing homologue. Two-dimensional NMR spectroscopy and modeling analysis gave evidence. . .

L8 ANSWER 4 OF 35 MEDLINE DUPLICATE 3
AB The MAPKKK **Ste11p** functions in three Saccharomyces cerevisiae MAPK cascades [the high osmolarity glycerol (HOG), pheromone response, and pseudohyphal/invasive growth pathways], but its. . . for Hog1p, allowed osmolarity-induced activation of the pheromone response pathway. This cross talk required the osmosensor Sho1p, as well as **Ste20p**, **Ste50p**, the pheromone response MAPK cascade (**Ste11p**, **Ste7p**, and Fus3p or Kss1p), and **Ste12p** but not **Ste4p** or the MAPK scaffold protein, **Ste5p**. The cross

talk in hog1 mutants [redacted]duced multiple responses of th[redacted]heromone response pathway: induction of a **FUS1**::lacZ reporter, morpholo[redacted]cal changes, and mating in **ste4** and **ste5** mutants. We suggest that Hog1p may prevent osmolarity-induced cross talk by inhibiting Sho1p, perhaps as part of a feedback control on the HOG pathway. We have also shown that **Ste20p** and **Ste50p** function in the Sho1p branch of the HOG pathway and that a second osmosensor in addition to Sho1p may activate **Ste11p**. Finally, we have found that pseudohyphal growth exhibited by wild-type (HOG1) strains depends on SHO1, suggesting that Sho1p may be. . .

L8 ANSWER 5 OF 35 MEDLINE DUPLICATE 4
AB . . . NOT proteins were found to co-immunoprecipitate with CCR4 and CAF1, and NOT1 co-purified with CCR4 and CAF1 through three chromatographic **steps** in a complex estimated to be 1.2x10(6) Da in size. Mutations in the NOT genes affected many of the same. . . derepression, defective cell wall integrity and increased sensitivity to monoand divalent ions. Similarly, ccr4, caf1 and dbf2 alleles negatively regulated **FUS1**-lacZ expression, as do defects in the NOT genes. These results indicate that the NOT proteins are physically and functionally part. . .

L8 ANSWER 6 OF 35 MEDLINE DUPLICATE 5
AB Basal and induced transcription of pheromone-dependent genes is regulated in a cell cycle-dependent way. **FUS1**, a gene strongly induced after pheromone treatment, shows high mRNA levels in mitosis and early G1 phase of the cell. . . CLN2 overexpression represses Fus3 kinase activity, independently of the phosphatase Msg5. Additionally, we show that the activity of the MEK **Ste7** also fluctuates during the cell cycle. Increased Cln2 levels repress the ability of hyperactive **STE11** alleles to induce the pathway. G protein-independent activation of **Ste11** caused by an rga1 pbs2 mutation is resistant to high levels of Cln2 kinase. Therefore our results suggest that Cln2-dependent repression of the mating pathway occurs at the level of **Ste11**.

L8 ANSWER 7 OF 35 MEDLINE DUPLICATE 6
AB . . . cells agglutinate and make contact but fail to undergo cell fusion. The chs5 mating defect can be partially rescued by **FUS1** and/or FUS2, two genes which have been implicated previously in cell fusion, but not by FUS3. In addition, mating efficiency is much lower in **fus1** fus2 x chs5 than in **fus1** fus2 x wild type crosses. Our results indicate that Chs5p plays an important role in the cell fusion **step** of mating.

L8 ANSWER 8 OF 35 MEDLINE DUPLICATE 7
AB . . . Ras2, stimulates both filamentous growth and expression of a transcriptional reporter FG(TyA)::lacZ but does not induce the mating pathway reporter **FUS1**::lacZ. This induction depends upon elements of the conserved mitogen-activated protein kinase (MAPK) pathway that is required for both filamentous growth and mating, two distinct morphogenetic events. Full induction requires **Ste20** (homolog of mammalian p65PAK protein kinases), **Ste11** [an MEK kinase (MEKK) or MAPK kinase (MEK) kinase], **Ste7** (MEK or MAPK kinase), and the transcription factor **Ste12**. Moreover, the Rho family protein Cdc42, a conserved morphogenetic G protein, is also a potent regulator of filamentous growth and FG(TyA)::lacZ expression in S. cerevisiae. Stimulation of both filamentous growth and FG(TyA)::lacZ by Cdc42 depends upon **Ste20**. In addition, dominant negative CDC42Ala118 blocks RAS2val19 activation, placing Cdc42 downstream of Ras2. Our results suggest that filamentous growth in. . .

L8 ANSWER 9 OF 35 MEDLINE DUPLICATE 8
AB . . . pathway is mediated by two G protein-linked receptors, each of which is expressed only in its specific cell type. The **STE3DAF** mutation results in inappropriate expression of the a-factor receptor in

MATa cells. Expressi[redacted] of this receptor in the inappr[redacted]iate cell type confers resistance to pheromone-induced G1 arrest, a p[redacted]enomenon that we have termed receptor inhibition. The ability of **STE3DAF** cells to cycle in the presence of pheromone was found to correlate with reduced phosphorylation of the cyclin-dependent kinase inhibitor Far1p. Measurement of Fus3p mitogen-activated protein (MAP) kinase activity in wild-type and **STE3DAF** cells showed that induction of Fus3p activity was the same in both strains at times of up to 1 h after pheromone treatment. However, after 2 or more hours, Fus3p activity declined in **STE3DAF** cells but remained high in wild-type cells. The level of inducible **FUS1** RNA paralleled the changes seen in Fus3p activity. Short-term activation of the Fus3p MAP kinase is therefore
sufficient for the. . . receptor inhibition is not simply a result of weak signaling but rather acts selectively at late times during the response. **STE3DAF** was found to inhibit the pheromone response pathway at a **step** between the G beta subunit and **Ste5p**, the scaffolding protein that binds the components of the MAP kinase phosphorylation cascade. Overexpression of **Ste20p**, a kinase thought to act between the G protein and the MAP kinase cascade, suppressed the **STE3DAF** phenotype. These findings are consistent with a model in which receptor inhibition acts by blocking the signaling pathway downstream of G protein dissociation and upstream of MAP kinase cascade activation, at a **step** that could directly involve **Ste20p**.

L8 ANSWER 10 OF 35 MEDLINE DUPLICATE 9
AB . . . genes involved in Saccharomyces cerevisiae mating were found to fluctuate during the cell cycle. In the absence of a functional **Ste12** transcription factor, both the levels and the cell cycle pattern of expression of these genes were affected. **FUS1** and AGA1 levels, which are maximally expressed only in G1-phase cells, were strongly reduced in **ste12**- cells. The cell cycle transcription pattern for FAR1 was changed in **ste12**- cells: the gene was still significantly expressed in G2/M, but transcript levels were strongly reduced in G1 phase, resulting in. . . resulted in increased levels of FAR1 transcription. The pattern of cell cycle-regulated transcription of FAR1 could involve combinatorial control of **Ste12** and Mcm1. Forced G1 expression of FAR1 from the GAL1 promoter resorted the ability to arrest in response to pheromone in **ste12**-cells. This indicates that transcription of FAR1 in the G1 phase is essential for accumulation of the protein and for pheromone-induced. . .

L8 ANSWER 11 OF 35 MEDLINE DUPLICATE 10
AB . . . yeast strains expressing the A2a adenosine receptor was elicited via activation of the yeast pheromone-response pathway. Induction of a pheromone-responsive **FUS1**-HIS3 reporter gene in far1 his3 cells permits cell growth in medium lacking histidine. The sensitivity of the bioassay was increased by deletion of the **STE2** gene, which encodes the yeast alpha-mating pheromone receptor. The growth response was
dose dependent, and agonists of varying affinities displayed. . .

L8 ANSWER 12 OF 35 MEDLINE DUPLICATE 11
AB . . . transduction.) AKR1 could serve as a multicopy suppressor of the lethality caused by either loss of GPA1 or overexpression of **STE4**, which encodes the G beta subunit of this G protein, suggesting that pheromone signaling is inhibited by overexpression of Akr1p.. . . in AKR1 displayed synthetic lethality with a weak allele of GPA1 and led to increased expression of the pheromone-inducible gene **FUS1**, suggesting that Akr1p normally (and not just when overexpressed) inhibits signaling. In contrast, deletion of BEM1 resulted in decreased expression of **FUS1**, suggesting that Bem1p normally facilitates pheromone signaling. During a screen for proteins that displayed two-hybrid interactions with Akr1p, we identified **Ste4p**, raising the possibility that an interaction between Akr1p and **Ste4p** contributes to proper regulation of the pheromone response pathway.

L8 ANSWER 13 OF 35 MEDLINE DUPLICATE 12

AB Pheromone signalling in Saccharomyces cerevisiae is mediated by the **STE4-STE18** G-protein beta gamma subunits. A possible target for the subunits is **Ste20p**, whose structural homolog, the serine/threonine kinase PAK, is activated by GTP-binding p21s Cdc42 and Rac1. The putative Cdc42p-binding domain of **Ste20p**, expressed as a fusion protein, binds human and yeast GTP-binding Cdc42p. Cdc42p is required for alpha-factor-induced activation of **FUS1**.cdc24ts strains defective for Cdc42p GDP/GTP exchange show no pheromone induction at restrictive temperatures but are partially rescued by overexpression of Cdc42p, which is potentiated by Cdc42p12V mutants. Epistatic analysis indicates that CDC24 and CDC42 lie between **STE4** and **STE20** in the pathway. The two-hybrid system revealed that **Ste4p** interacts with Cdc24p. We propose that Cdc42p plays a pivotal role both in polarization of the cytoskeleton and in pheromone.

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L8 ANSWER 14 OF 35 MEDLINE DUPLICATE 13

AB In Schizosaccharomyces pombe, the **fus1** mutation blocks conjugation at a point after cell contact and agglutination. The cell walls separating the mating partners are not degraded, which prevents cytoplasmic fusion. In order to investigate the molecular mechanism of conjugation, we cloned the **fus1** gene and found that it is capable of encoding a 1,372-amino-acid protein with no significant similarities to other known proteins. Expression of the **fus1** gene is regulated by the developmental state of the cells. Transcription is induced by nitrogen starvation and requires a pheromone signal in both P and M cell types. Consequently, mutants defective in the pheromone response pathway fail to induce **fus1** expression. The **ste11** gene, which encodes a transcription factor controlling expression of many genes involved in sexual differentiation, is also required for transcription of **fus1**. Furthermore, deletion of two potential **Ste11** recognition sites in the **fus1** promoter region abolished transcription, and expression could be restored when we inserted a different **Ste11** site from the mat1-P promoter. Since this element was inverted relative to the **fus1** element, we conclude that activation of transcription by **Ste11** is independent of orientation. Although the **fus1** mutant has a phenotype very similar to that of Saccharomyces cerevisiae **fus1** mutants, the two proteins appear to have different roles in the process of cell fusion. Budding yeast **Fus1** is a typical membrane protein and contains an SH3 domain. Fission yeast **Fus1** has no features of a membrane protein, yet it appears to localize to the projection tip. A characteristic proline-rich potential. . .

L8 ANSWER 15 OF 35 MEDLINE DUPLICATE 14

AB . . . RanBP1 on rcc1- cells was confirmed by the finding that overproduction of RanBP1 induces significant levels of expression of a **FUS1**-lacZ gene and an increase in mating efficiencies in a **ste3**, pheromone receptor-deficient yeast mutant. This phenotype is similar to the srm1, a mutant isolated as a suppressor that restores mating. . .

L8 ANSWER 16 OF 35 MEDLINE DUPLICATE 15

AB . . . a protein with structural similarity to MAP kinase kinases. Expression of this gene in Saccharomyces cerevisiae complements disruption of the **Ste7** MAP kinase kinase required for both mating in haploid cells and pseudohyphal growth in diploids. However, Hst7 expression does not. . . Mkk1 and Mkk2 MAP kinase kinases required for proper cell wall biosynthesis. Intriguingly, HST7 acts as a hyperactive allele of **STE7**; expression of Hst7 activates the mating pathway even in the absence of upstream signaling components including the **Ste7** regulator **Ste11**, elevates the basal level of the pheromone-inducible **FUS1** gene, and amplifies the pseudohyphal growth response in diploid cells. Thus Hst7 appears to be at least

partially independent of upstream activators or regulators, but selective in its activity on downstream target MAP kinases. Creation of Hst7/**Ste7** hybrid proteins revealed that the C-terminal two-thirds of Hst7, which contains the protein kinase domain, is sufficient to confer this. . .

L8 ANSWER 17 OF 35 MEDLINE DUPLICATE 16
AB The **STE4** gene encodes the beta subunit of a heterotrimeric G protein that is an essential component of the pheromone signal transduction pathway. To identify downstream component(s) of **Ste4**, we sought pseudo-revertants that restored mating competence to **ste4** mutants. The suppressor mot2 was isolated as a recessive mutation that restored conjugational competence to a temperature-sensitive **ste4** mutant and simultaneously conferred a temperature-sensitive growth phenotype. The MOT2 gene encodes a putative zinc finger protein, the deletion of which resulted in temperature-sensitive growth, increased expression of **FUS1** in the absence of pheromones, and suppression of a deletion of the alpha-factor receptor. On the other hand, **sterility** resulting from deletion of **STE4** was not suppressed by the mot2 deletion. These phenotypes are similar to those associated with temperature-sensitive mutations in CDC36 and. . .

L8 ANSWER 18 OF 35 MEDLINE DUPLICATE 17
AB . . . transmission of the pheromone response signal. The DAF2-2 mutation has two effects on the expression of a pheromone inducible gene, **FUS1**. In DAF2-2 cells, **FUS1** RNA is present at an increased basal level but is no longer fully inducible by pheromone. Cloning of DAF2-2 revealed that it is an allele of **STE3**, the gene encoding the a-factor receptor. **STE3** is normally an alpha-specific gene, but is inappropriately expressed in a cells carrying a **STE3DAF2**-2 allele. The two effects of **STE3DAF2**-2 alleles on the pheromone response pathway are the result of different functions of the receptor. The increased basal level of **FUS1** RNA is probably due to stimulation of the pathway by an autocrine mechanism, because it required at least one of. . . a-factor. Suppression of a null allele of the G alpha subunit gene, the phenotype associated with the inhibitory function of **STE3**, was independent of a-factor. This suppression was also observed when the wild-type **STE3** gene was expressed in a cells under the control of an inducible promoter. Inappropriate expression of **STE2** in alpha cells was able to suppress a point mutation, but not a null allele, of the G alpha subunit. . .

L8 ANSWER 19 OF 35 MEDLINE DUPLICATE 18
AB SCG1/GPA1, **STE4** and **STE18** encode the alpha, beta and gamma components of the G protein involved in mating pheromone signal transduction in Saccharomyces cerevisiae. Responses, including G1 arrest and expression of genes such as **FUS1**, are activated by beta gamma, which is negatively controlled by alpha(GDP). We previously demonstrated that overexpression of Scg1 suppresses responses. . . scg1-null mutant. Effects were attributed to sequestration of beta gamma. We now show that effects on growth rate, morphology and **FUS1** expression are consistent with this model. The **STE4HPL** allele causes dominant activation of the response pathway, and is presumed to encode a beta subunit insensitive to control by alpha(GDP). Scg1 overexpression suppresses the growth arrest due to **STE4HPL;** normal alpha-factor responses and fertility are restored. A model based on sequestration of beta gamma reconciles this result with the. . . responses and mating in wild-type cells. A G alpha i hybrid also restores growth and allows inefficient mating in the **STE4HPL** strain.

L8 ANSWER 20 OF 35 MEDLINE DUPLICATE 19
AB . . . presumptive protein kinases of Schizosaccharomyces pombe, byr2, byr1, and spk1 that are structurally related to protein kinases of Saccharomyces cerevisiae, **STE11**, **STE7**, and FUS3, respectively, are also functionally related. In some cases, introduction

of the heterologous [redacted]tein kinase into a mutant was [redacted]fficient for complementation. In other cases (as in a **ste11**- mutant of S. cerevisiae), expression of two S. pombe protein kinases (byr2 and byr1) was required to observe complementation, suggesting. . . sporulation and conjugation and in S. cerevisiae as restoration of conjugation, pheromone-induced cell cycle arrest, and pheromone-induced transcription of the **FUS1** gene. We also show that the S. pombe kinases bear a similar relationship to the mating pheromone receptor apparatus as. . . two evolutionarily distant yeasts despite an apparently significant difference in function of the heterotrimeric G proteins. We suggest that the **STE11**/byr2, **STE7**/byr1, and FUS3/spk1 kinases comprise a signal transduction module that may be conserved in higher eukaryotes. Consistent with this hypothesis, we. . .

L8 ANSWER 21 OF 35 MEDLINE DUPLICATE 20
AB . . . improved response to Saccharomyces kluyveri alpha-factor were identified and sequenced. Mutants were isolated from cells bearing randomly mutagenized receptor gene (**STE2**) plasmids by an in vivo screen. Five mutations lead to substitutions in hydrophobic segments in the core of the receptor. . . arrest initially, but then recovered more efficiently (S145L-S219L). One mutant (L255S-S288P) conferred positive pheromone responses to alpha-factor as assayed by **FUS1**-lacZ reporter induction, but did not display growth arrest. The hydrophobic receptor core thus appears to control activation by some ligands. . .

L8 ANSWER 22 OF 35 MEDLINE DUPLICATE 21
AB A new gene, **STE50**, which plays an essential role in cell differentiation in Saccharomyces cerevisiae was detected and analysed. **STE50** expression is not cell type-specific and its expression in MATa and MAT alpha cells is unaffected by pheromones. When present on a high copy number plasmid, **STE50** causes supersensitivity to alpha-pheromone, and increases the level of alpha-pheromone-induced transcription of **FUS1** in haploid a cells. Mutants bearing either of the two gene disruptions, **ste50**-1 or **ste50**-2, are **sterile** and have a modulated sensitivity to alpha-pheromone. The overexpression of **STE4** (G beta) in wild-type cells elicits a constitutive growth arrest signal, however this phenotype is suppressed by a C-terminal truncation mutation in **STE50** (**ste50**-2). In contrast, the constitutive activation of the pheromone response pathway caused by disruption of GPA1 (G alpha) is not suppressed in **ste50** -2 mutants. The **ste50**-2 mutation partially suppresses the desensitisation defect of the sst2-1 mutation, and the resulting **ste50**-2 sst2-1 mutants restore fertility. Our results indicate that the **ste50**-2 mutant may have a defect in adaptation (hyperadaptation), and suggest a possible interaction of **STE50**-2 with the G alpha subunit of the G protein.

L8 ANSWER 23 OF 35 MEDLINE DUPLICATE 22
AB . . . 1991]). Genes regulated by stimuli as diverse as external signals (PHO5), cell differentiation processes (SPO11 and SPO13), cell type (RME1, **FUS1**, HO, TY2, **STE6**, **STE3**, and BAR1), and genes whose regulatory signals remain unknown (TRK2) depend on RPD1 to achieve maximal states of transcriptional regulation.. . .

L8 ANSWER 24 OF 35 MEDLINE DUPLICATE 23
AB . . . cells. The concentration of alpha-factor required for a half-maximal inhibition was comparable to that required for the induction of the **FUS1** gene. Strains containing a disruption in **ste2** or **ste12** or temperature-sensitive mutations in **ste4**, **ste7**, or **ste11** continued to divide and to accumulate glycogen in the presence of alpha-factor. In contrast, inhibition of glycogen occurred upon exposure. . .

L8 ANSWER 25 OF 35 MEDLINE DUPLICATE 24

AB . . . respond to [redacted]eromone throughout the cycle e[redacted]though there is cell cycle modulation of the expression of two pherom[redacted]e-inducible genes, **FUS1** and **STE2**. Both of these genes are expressed less efficiently near or just after the START point of the cell cycle in response to alpha-factor. For **STE2**, the basal level of expression is modulated in the same manner.

L8 ANSWER 26 OF 35 MEDLINE DUPLICATE 25

AB The **FUS1** gene of Saccharomyces cerevisiae is transcribed in a and alpha cells, not in a/alpha diploids, and its transcription increases dramatically when haploid cells are exposed to the appropriate mating pheromone. In addition, **FUS1** transcription is absolutely dependent on **STE4**, **STE5**, **STE7**, **STE11**, and **STE12**, genes thought to encode components of the pheromone response pathway. We now have determined that the pheromone response element (PRE), which occurs in four copies within the **FUS1** upstream region, functions as the **FUS1** upstream activation sequence (UAS) and is responsible for all known aspects of **FUS1** regulation. In particular, deletion of 55 bp that includes the PREs abolished all transcription, and a 139-bp fragment that includes the PREs conferred **FUS1**-like expression to a CYC1-lacZ reporter gene. Moreover, three or four copies of a synthetic PRE closely mimicked the activity conferred. . . . even a single copy of PRE conferred a trace of activity that was haploid specific and pheromone inducible. In the **FUS1** promoter context, four copies of the synthetic PRE inserted at the site of the 55-bp deletion restored full **FUS1** transcription. Sequences upstream and downstream from the PRE cluster were important for maximal PRE-directed expression but, by themselves, did not have UAS activity. Other yeast genes with PREs, e.g., **STE2** and BAR1, are more modestly inducible and have additional UAS elements contributing to the overall activity. In the **FUS1** promoter, the PREs apparently act alone to confer activity that is highly stimulated by pheromone.

L8 ANSWER 27 OF 35 MEDLINE DUPLICATE 26

AB The yeast GPA1, **STE4**, and **STE18** genes encode proteins homologous to the respective alpha, beta and gamma subunits of the mammalian G protein complex which appears to mediate the response to mating pheromones. Overexpression of the **STE4** protein by the galactose-inducible GAL1 promoter caused activation of the pheromone response pathway which resulted in cell-cycle arrest in late G1 phase and induction of the **FUS1** gene expression, thereby suppressing the **sterility** of the receptor-less mutant delta **ste2**. Disruption of **STE18**, in turn, suppressed activation of the pheromone response induced by overexpression of **STE4**, suggesting that the **STE18** product is required for the **STE4** action. However, overexpression of both the **STE4** and **STE18** proteins did not generate a stronger pheromone response than overexpression of **STE4** in the presence of wild-type levels of **STE18**. These results suggest that the beta subunit is the limiting component for the pheromone response and support the idea that beta and gamma subunits act as a positive regulator. Furthermore, overexpression of GPA1 prevented cell-cycle arrest but not **FUS1** induction mediated by overexpression of **STE4**. This implies that the alpha subunit acts as a negative regulator presumably through interacting with beta and gamma subunits in. . .

L8 ANSWER 28 OF 35 MEDLINE DUPLICATE 27

AB . . . more detailed investigation of pheromone response in the end1 mutant reveals that one aspect of the early response (induction of **FUS1**) is as defective as late responses (cell cycle arrest and projection formation). In contrast, another measure of the early response (induction of **STE2**) is normal. These data suggest that the biogenesis of a functional vacuole is necessary for optimal response to pheromone.

L8 ANSWER 29 OF 35 MEDLINE DUPLICATE 28

AB The Saccharomyces cerevisiae GPA1, **STE4**, and **STE18** genes encode products homologous to mammalian G-protein alpha, beta, and gamma subunits, respectively. All three genes function in the transduction. . . pheromone in haploid cells. To characterize more completely the role of these genes in mating, we have conditionally overexpressed GPA1, **STE4**, and **STE18**, using the galactose-inducible GAL1 promoter. Overexpression of **STE4** alone, or **STE4** together with **STE18**, generated a response in haploid cells suggestive of pheromone signal transduction: arrest in G1 of the cell cycle, formation of cellular projections, and induction of the pheromone-inducible transcript **FUS1** 25- to 70-fold. High-level **STE18** expression alone had none of these effects, nor did overexpression of **STE4** in a MATa/alpha diploid. However, **STE18** was essential for the response, since overexpression of **STE4** was unable to activate a response in a **ste18** null strain. GPA1 hyperexpression suppressed the phenotype of **STE4** overexpression. In addition, cells that overexpressed GPA1 were more resistant to pheromone and recovered more quickly from pheromone than did.
. .

L8 ANSWER 30 OF 35 MEDLINE DUPLICATE 29
AB . . . mating pheromone. We demonstrate here that strains harboring temperature-sensitive mutations in CDC36 or CDC39 activate expression of the pheromone-inducible gene **FUS1** when shifted to nonpermissive temperature. We show further that cell-cycle arrest and induction of **FUS1** are dependent on known components of the mating factor response pathway, the **STE** genes. Thus, the G1-arrest phenotype of cdc36 and cdc39 mutants results from activation of the mating factor response pathway. The. . . required to block response in the absence of pheromone. Epistasis analysis of mutants defective in CDC36 or CDC39 and different **STE** genes demonstrates that activation requires the response pathway G protein and suggests that CDC36 and CDC39 products may control synthesis. . .

L8 ANSWER 31 OF 35 MEDLINE DUPLICATE 30
AB . . . alpha-factor in Saccharomyces cerevisiae MATa cells was identified and characterized genetically. Whereas wild-type cells induce a high level of the **FUS1** mRNA from a low baseline on exposure to alpha-factor, DAF2-2 cells were constitutive producers of an intermediate level of **FUS1** RNA; the level was increased only modestly by alpha-factor. **FUS1** constitutivity required **STE4**, **STE5** and **STE18**, but did not require **STE2**, the alpha-factor receptor gene. DAF2-2 suppressed the alpha-factor supersensitivity of a **STE2** C-terminal truncation, and suppressed lethality due to scg1 mutations. Thus DAF2-2 may act by uncoupling the signaling pathway from alpha-factor binding at some point in the pathway between Scg1 inactivation and the action of **Ste4**, **Ste5** and **Ste18**; this uncoupling might occur at the expense of partial constitutive activation of the pathway. DAF2-2 suppressed the unconditional cell-cycle arrest phenotype of a dominant "constitutive signaling" allele of **STE4** (**STE4Hpl**), although the constitutive **FUS1** phenotype of DAF2-2 was suppressed by **ste4** null mutations; therefore DAF2-2 may directly affect the performance of the **STE4 step**.

L8 ANSWER 32 OF 35 MEDLINE DUPLICATE 31
AB . . . To identify components of the signal transduction pathway, we sought pseudorevertants that restored mating competence to receptor mutants (MAT alpha **ste3**::LEU2). The suppressor srm1-1 was isolated as a recessive mutation that conferred temperature-sensitive growth to all strains and mating ability to MAT alpha **ste3**::LEU2 strains at the nonpermissive temperature. In addition, when srm1-1 mutants were shifted to the nonpermissive temperature, they exhibited two phenotypes characteristic of pheromone response, induction of **FUS1**

transcription and a[redacted]ulation of cells in the G1 pha[redacted]of the cell cycle.
The srm1-1 mutation also suppressed a deletion. . . cells. Together, these phenotypes suggest that the wild-type SRM1 product is a component of
the pheromone response pathway. Deletion of **STE4** or **STE5**, which are required in both haploid cell types for mating and response to
pheromone, was not suppressed by srm1-1, suggesting that the SRM1 product may function before the **STE4** and **STE5** products. SRM1 is an essential gene and is expressed in both haploid cell types as well as in the product. . .

L8 ANSWER 33 OF 35 MEDLINE DUPLICATE 32
AB The **ste** mutants (**ste2**, **ste4**, **ste5**, **ste7**, **ste11**, and **ste12**) are insensitive to mating factors and are, therefore, **sterile**. Roles of the **STE** gene products in the GPA1-mediated mating factor signaling pathway were studied by using **ste** gpa1 double mutants. Mating efficiency of a **ste2** mutant defective in the alpha-factor receptor increased 1,000-fold in a gpa1 background, while G1 arrest and aberrant morphology (shmoo) caused by gpa1 were not suppressed by **ste2**. Furthermore, the **steady**-state level of the **FUS1** transcript, which normally increases in response to mating factors, was also elevated when the GPA1 function was impaired. These results suggest that the GPA1 protein functions downstream of the **STE2** receptor. Conversely, the **sterility** of **ste4**, **ste5**, **ste7**, **ste11**, and **ste12** mutants was not suppressed by gpa1, but the lethal phenotype of gpa1 was suppressed by these **ste** mutations. Northern (RNA) blotting analysis revealed that the **ste7**, **ste11**, and **ste12** mutations caused reductions of 50 to 70% in the **steady**-state levels of the GPA1 transcript, while **ste4** had a slight effect and **ste5** had no effect. This implies that the suppression by **ste7**, **ste11**, and **ste12** could be due to reduced syntheses of additional components, including an effector, and that suppression by **ste4** and **ste5** may result from direct effects on the signaling pathway. The **STE4**, **STE5**, **STE7**, **STE11**, and **STE12** products, therefore, appear to specify components of the signal transduction machinery, directly or indirectly, which function together with or downstream. . .

L8 ANSWER 34 OF 35 MEDLINE DUPLICATE 33
AB . . . bearing either of two recessive mutations, sgp1 and sgp2, in combination with the disruption mutation, showed a cell type nonspecific **sterile** phenotype, yet expressed the major alpha-factor gene (MF alpha 1) as judged by the ability to express a MF alpha. . . GPA1 locus. The sgp2 mutation was not linked to GPA1 and was different from the
previously identified cell type nonspecific **sterile** mutations (**ste4**, **ste5**, **ste7**, **ste11** and **ste12**). sgp2 GPA1 cells showed a fertile phenotype, indicating that the mating defect caused by sgp2 is associated with the loss of GPA1 function. While expression of a **FUS1**-lacZ fusion gene was induced in wild-type cells by the addition of alpha-factor, mutants bearing sgp1 or sgp2 as well as gpa1::HIS3 constitutively expressed **FUS1**-lacZ. These observations suggest that GPA1 (SGP1) and SGP2 are involved in mating factor-mediated signal transduction, which causes both cell cycle arrest in the late G1 phase and induction of genes necessary for mating such as **FUS1**.

L8 ANSWER 35 OF 35 MEDLINE DUPLICATE 34
AB . . . cerevisiae whose expression is affected by cell type or by the mating pheromones. From this screen we identified a gene, **FUS1**, whose pattern of expression revealed interesting regulatory strategies and
whose product was required for efficient cell fusion during mating. Transcription of **FUS1** occurred only in a and alpha cells, not in

a/alpha cells, where [redacted] was repressed by a1 X alpha 2 [redacted] regulatory activity present uniquely in a/alpha cells. Transcription of **FUS1** showed an absolute requirement for the products of five **STE** genes, **STE4**, **STE5**, **STE7**, **STE11**, and **STE12**. Since the activators **STE4**, **STE5**, and **STE12** are themselves repressed by a1 X alpha 2, the failure to express **FUS1** in a/alpha cells is probably the result of a cascade of regulatory activities; repression of the activators by a1 X alpha 2 in turn precludes transcription of **FUS1**. In addition to regulation of **FUS1** by cell type, transcription from the locus increased 10-fold or more when a or alpha cells were exposed to the opposing mating pheromone. To investigate the function of the **Fus1** protein, we created **fus1** null mutants. In **fus1** X **fus1** matings, the cells of a mating pair adhered tightly and appeared to form zygotes. However, the zygotes were abnormal. Within. . . the conjugation bridge the contained a partition that prevented nuclear fusion and mixing of organelles. The predicted sequence of the **Fus1** protein (deduced from the **FUS1** DNA sequence) and subcellular fractionation studies with **Fus1**-beta-galactosidase hybrid proteins suggest that **Fus1** is a membrane or secreted protein. Thus, **Fus1** may be located at a position within the cell where it is poised to catalyze cell wall or plasma membrane. . .

```
=> d his

     (FILE 'HOME' ENTERED AT 11:39:18 ON 03 MAY 2000)

     FILE 'MEDLINE, BIOSIS' ENTERED AT 11:39:28 ON 03 MAY 2000
L1           155 S FUS1
L2             0 S FUS1-STE4
L3             0 S FUS1-STE5
L4             0 S FUS1 SAME STE4
L5             0 S FUS1-STE?
L6       1424375 S STE?
L7            69 S L6 (S) L1
L8            35 DUP REM L7 (34 DUPLICATES REMOVED)

=> s fus1-lacz

L9            22 FUS1-LACZ

=> s l8 (s) (fusion or hybride)

PROXIMITY OPERATOR LEVEL NOT CONSISTENT WITH
FIELD CODE - 'AND' OPERATOR ASSUMED 'L28 (S) '
L10            5 L8 (S) (FUSION OR HYBRIDE)

=> d kwic 1-5

'L-5' IS NOT A VALID FORMAT FOR FILE 'MEDLINE'

The following are valid formats:

The default display format is BIB.

ABS ---- AB
ALL ---- AN, DN, TI, CM, AU, CS, NC, SO, CY, DT, LA, FS, OS, EM, EW,
         AB, CT, ST, RN, CN, NA, GEN
BIB ---- AN, DN, TI, CM, AU, CS, NC, SO, CY, DT, LA, FS, OS, EM, EW
CBIB --- AN, DN, TI, CM, AU, CS, NC, SO, CY, DT, LA, FS, OS, EM, EW
DALL --- ALL, delimited for post processing
IABS --- ABS, with a text label
IALL --- ALL, indented with text labels
IBIB --- BIB, indented with text lables
IND ---- CT, ST, RN, CN, NA, GEN
SAM ---- TI, CM, CT, ST, RN, CN, NA, GEN
```

TRI ---- TI, CM, CT, ST, [redacted] CN, NA, GEN
TRIAL -- TI, CM, CT, ST, RN, CN, NA, GEN
HIT ---- All fields containing hit terms
HITIND - IND
KWIC --- All hit terms plus 20 words on either side
OCC ---- List of display fields containing hit terms

Hit terms will be highlighted in all available fields except CM and PY.

To display a particular field or fields, enter the display field codes. For a list of display field codes, enter 'HELP DFIELDS' at an arrow prompt (=>). Examples of formats include: 'BIB'; 'AB'; 'SO,ST'. You may specify the format fields in any order, and the information will be displayed in the same order as the format specification.

The same formats (except for HIT, HITIND, KWIC, and OCC) may be used with the DISPLAY ACC command to display the record for a specified Accession Number.
ENTER DISPLAY FORMAT (BIB):end

=> d his

(FILE 'HOME' ENTERED AT 11:39:18 ON 03 MAY 2000)

FILE 'MEDLINE, BIOSIS' ENTERED AT 11:39:28 ON 03 MAY 2000
L1 155 S FUS1
L2 0 S FUS1-STE4
L3 0 S FUS1-STE5
L4 0 S FUS1 SAME STE4
L5 0 S FUS1-STE?
L6 1424375 S STE?
L7 69 S L6 (S) L1
L8 35 DUP REM L7 (34 DUPLICATES REMOVED)
L9 22 S FUS1-LACZ
L10 5 S L8 (S) (FUSION OR HYBRIDE)

=> d kwic l10

L10 ANSWER 1 OF 5 MEDLINE
AB . . . synthesis. Analysis of mating mixtures of chs5 cells reveals that
cells agglutinate and make contact but fail to undergo cell **fusion**. The chs5 mating defect can be partially rescued by **FUS1** and/or FUS2, two genes which have been implicated previously in cell **fusion**, but not by FUS3. In addition, mating efficiency is much lower in **fus1** fus2 x chs5 than in **fus1** fus2 x wild type crosses. Our results indicate that Chs5p plays an important role in the cell **fusion step** of mating.

=> d kwic 2-4

L10 ANSWER 2 OF 5 MEDLINE
AB Pheromone signalling in Saccharomyces cerevisiae is mediated by the **STE4-STE18** G-protein beta gamma subunits. A possible target for the subunits is **Ste20p**, whose structural homolog, the serine/threonine kinase PAK, is activated by GTP-binding p21s Cdc42 and Rac1. The putative Cdc42p-binding domain of **Ste20p**, expressed as a **fusion** protein, binds human and yeast GTP-binding Cdc42p. Cdc42p is required for alpha-factor-induced activation of **FUS1**.cdc24ts strains defective for Cdc42p GDP/GTP exchange show no pheromone induction at restrictive temperatures but are partially rescued by overexpression of Cdc42p, which is potentiated by Cdc42p12V mutants.

Epistatic analysis indicates that CDC24 and CDC42 lie between **STE4** and **STE20** in the pathway. The two-hybrid system revealed that **Ste4p** interacts with Cdc24p. We propose that Cdc42p plays a pivotal role both in polarization of the cytoskeleton and in pheromone.

.

L10 ANSWER 3 OF 5 MEDLINE

AB In Schizosaccharomyces pombe, the **fus1** mutation blocks conjugation at a point after cell contact and agglutination. The cell walls separating the mating partners are not degraded, which prevents cytoplasmic **fusion**. In order to investigate the molecular mechanism of conjugation, we cloned the **fus1** gene and found that it is capable of encoding a 1,372-amino-acid protein with no significant similarities to other known proteins. Expression of the **fus1** gene is regulated by the developmental state of the cells. Transcription is induced by nitrogen starvation and requires a pheromone signal in both P and M cell types. Consequently, mutants defective in the pheromone response pathway fail to induce **fus1** expression. The **ste11** gene, which encodes a transcription factor controlling expression of many genes involved in sexual differentiation, is also required for transcription of **fus1**. Furthermore, deletion of two potential **Ste11** recognition sites in the **fus1** promoter region abolished transcription, and expression could be restored when we inserted a different **Ste11** site from the mat1-P promoter. Since this element was inverted relative to the **fus1** element, we conclude that activation of transcription by **Ste11** is independent of orientation. Although the **fus1** mutant has a phenotype very similar to that of Saccharomyces cerevisiae **fus1** mutants, the two proteins appear to have different roles in the process of cell **fusion**. Budding yeast **Fus1** is a typical membrane protein and contains an SH3 domain. Fission yeast **Fus1** has no features of a membrane protein, yet it appears to localize to the projection tip. A characteristic proline-rich potential. . .

L10 ANSWER 4 OF 5 MEDLINE

AB . . . bearing either of two recessive mutations, sgp1 and sgp2, in combination with the disruption mutation, showed a cell type nonspecific **sterile** phenotype, yet expressed the major alpha-factor gene (MF alpha 1) as judged by the ability to express a MF alpha 1-lacZ **fusion** gene. The sgp1 mutation was closely linked to gpa1::HIS3 and probably occurred at the GPA1 locus. The sgp2 mutation was not linked to GPA1 and was different from the previously identified cell type nonspecific **sterile** mutations (**ste4**, **ste5**, **ste7**, **ste11** and **ste12**). sgp2 GPA1 cells showed a fertile phenotype, indicating that the mating defect caused by sgp2 is associated with the loss of GPA1 function. While expression of a **FUS1**-lacZ **fusion** gene was induced in wild-type cells by the addition of alpha-factor, mutants bearing sgp1 or sgp2 as well as gpa1::HIS3 constitutively expressed **FUS1**-lacZ. These observations suggest that GPA1 (SGP1) and SGP2 are involved in mating factor-mediated signal transduction, which causes both cell cycle arrest in the late G1 phase and induction of genes necessary for mating such as **FUS1**.

=> s bar1

L11 46 BAR1

=> s bar1-ste?

L12 0 BAR1-STE?

=> s fus1-kss?

L13 0 FUS1-KSS?

=> s bar1-kss?

L14 0 BAR1-KSS?

=> s fus?-ste?

L15 392 FUS?-STE?

=> s l15 (s) (fusion or hybr?)

L16 243 L15 (S) (FUSION OR HYBR?)

=> d kwic 1-10

L16 ANSWER 1 OF 243 MEDLINE
AB . . . were selectively enriched in the detergent-resistant glycosphingolipids and cholesterol-rich membranes (rafts). Isolated rafts could infect the cell through a membrane **fusion step** and thus contained all of the components required to create a functional virion. However, they could be distinguished from the. . .

L16 ANSWER 2 OF 243 MEDLINE
AB . . . transporter GLUT4 has similarities to regulated synaptic transmission. A recent study has now identified a key regulated component of the **fusion step** in the exocytosis of these GLUT4-containing vesicles.

L16 ANSWER 3 OF 243 MEDLINE
AB . . . bundles. Over the next few days, the stereocilia become progressively more disorganised and fuse together. Practically all hair cells show **fused stereocilia** by 3 days after birth, and there is extensive stereocilia **fusion** by 7 days. By 20 days, giant stereocilia are observed on top of the hair cells. At 1 and 3. .
.
controls take up the membrane dye FM1-43, suggesting that endocytosis occurs in mutant hair cells. One possible model for the **fusion** is that myosin VI may be involved in anchoring the apical hair cell membrane to the underlying actin-rich cuticular plate,. . . and in the absence of normal myosin VI this apical membrane will tend to pull up between stereocilia, leading to **fusion**. Copyright 1999 Academic Press.

L16 ANSWER 4 OF 243 MEDLINE
AB . . . cells suggest that the calmodulin-MLCK system plays an essential role in the ATP-requiring priming stage but not in the Ca2(+)-triggered **fusion step** in the exocytotic process in bovine adrenal chromaffin cells.

L16 ANSWER 5 OF 243 MEDLINE
AB Members of the syntaxin protein family participate in the docking-**fusion step** of several intracellular vesicular transport events. Tlg1p has been identified as a nonessential protein required for efficient endocytosis as well. . .

L16 ANSWER 6 OF 243 MEDLINE
AB . . . alkaline phosphatase and aminopeptidase I to the vacuole. The v-SNARE Nyv1p forms a SNARE complex with Vam3p in homotypic vacuolar **fusion**; however, we find that Nyv1p is not required for any of the three biosynthetic pathways to the vacuole. v-SNAREs were. . . to the prevacuolar compartment and with Sed5p in retrograde traffic to the cis-Golgi. The ability of Vti1p to mediate multiple **fusion steps** requires additional proteins to ensure specificity in membrane traffic.

L16 ANSWER 7 OF 243 MEDLINE
AB . . . depolarizations by elevated extracellular [K+]. Findings were interpreted as sequential transitions between the previously characterized

pools of vesicles preceding the **fusion step**. The observed approach of vesicles to their docking sites was not explained in terms of free diffusion: most vesicles moved. . . binding sites at the plasma membrane. Vesicle mobility at the membrane was low, such that the sites of docking and **fusion** were in close vicinity. Both the rim region and confined areas in the centre of the footprint region were the. . .

L16 ANSWER 8 OF 243 MEDLINE
AB . . . gp120 and gp41. The extraviral portion (ectodomain) of gp41 contains an alpha-helical domain that likely represents the core of the **fusion**-active conformation of the molecule. Here we report the identification and characterization of a minimal, autonomous folding subdomain that retains key. . . disulfide-bonded loop sequence. N34(L6)C28 forms a highly thermostable, alpha-helical trimer. Point mutations within the envelope protein complex that abolish membrane **fusion** and HIV-1 infectivity also impede the formation of the N34(L6)C28 core. Moreover, N34(L6)C28 is capable of inhibiting HIV-1 envelope-mediated membrane **fusion**. Taken together, these results indicate that the N34(L6)C28 core plays a direct role in the membrane **fusion step** of HIV-1 infection and thus provides a molecular target for the development of antiviral pharmaceutical agents.

L16 ANSWER 9 OF 243 MEDLINE
AB . . . Vesicles undergo sequential transitions between several states of differing fluorescence intensity and mobility. The transitions are reversible, except for the **fusion step**, and even in nonstimulated conditions the vesicles change states in a dynamic equilibrium. Stimulation selectively speeds up the three forward. . . mobility in all three dimensions upon approach of the plasma membrane. Their movement is directed and targeted to the docking **fusion** sites. Sites of vesicle docking and exocytosis are distributed non-uniformly over the studied "footprint" region of the cell. While some areas are the sites of repeated vesicle docking and **fusion**, others are completely devoid of spots. Vesicular mobility at the membrane is confined, as if the vesicle were imprisoned in. . .

L16 ANSWER 10 OF 243 MEDLINE
AB Glucose metabolism is essential for successful gamete **fusion** in the mouse. Although the metabolic activity of the oocyte does not appear to play a significant role in the **fusion step**, the metabolic role of the spermatozoon is not known. The aim of this study was therefore to characterize the role. . .

=> s fus2

L17 26 FUS2

=> s fus2-ste?

L18 0 FUS2-STE?

=> s fus1 promoter

L19 4 FUS1 PROMOTER

=> dup rem l19

PROCESSING COMPLETED FOR L19
L20 2 DUP REM L19 (2 DUPLICATES REMOVED)

=> d ibib abs 1-2

L20 ANSWER 1 OF 2 MEDLINE DUPLICATE 1
ACCESSION NUMBER: 95311967 MEDLINE

DOCUMENT NUMBER: 9531[illegible]
TITLE: Characterization of fus1 of Schizosaccharomyces pombe: a developmentally controlled function needed for conjugation.
AUTHOR: Petersen J; Weilguny D; Egel R; Nielsen O
CORPORATE SOURCE: Department of Genetics, University of Copenhagen, Denmark.
SOURCE: MOLECULAR AND CELLULAR BIOLOGY, (1995 Jul) 15 (7) 3697-707.
Journal code: NGY. ISSN: 0270-7306.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
OTHER SOURCE: GENBANK-L37838
ENTRY MONTH: 199509

AB In Schizosaccharomyces pombe, the fus1 mutation blocks conjugation at a point after cell contact and agglutination. The cell walls separating the mating partners are not degraded, which prevents cytoplasmic fusion. In order to investigate the molecular mechanism of conjugation, we cloned the fus1 gene and found that it is capable of encoding a 1,372-amino-acid protein with no significant similarities to other known proteins. Expression of the fus1 gene is regulated by the developmental state of the cells. Transcription is induced by nitrogen starvation and requires a pheromone signal in both P and M cell types. Consequently, mutants defective in the pheromone response pathway fail to induce fus1 expression. The ste11 gene, which encodes a transcription factor controlling expression of many genes involved in sexual differentiation, is also required for transcription of fus1. Furthermore, deletion of two potential Ste11 recognition sites in the **fus1 promoter** region abolished transcription, and expression could be restored when we inserted a different Ste11 site from the mat1-P promoter. Since this element was inverted relative to the fus1 element, we conclude that activation of transcription by Ste11 is independent of orientation. Although the fus1 mutant has a phenotype very similar to that of Saccharomyces cerevisiae fus1 mutants, the two proteins appear to have different roles in the process of cell fusion. Budding yeast Fus1 is a typical membrane protein and contains an SH3 domain. Fission yeast Fus1 has no features of a membrane protein, yet it appears to localize to the projection tip. A characteristic proline-rich potential SH3 binding site may mediate interaction with other proteins.

L20 ANSWER 2 OF 2 MEDLINE DUPLICATE 2
ACCESSION NUMBER: 91246161 MEDLINE
DOCUMENT NUMBER: 91246161
TITLE: Pheromone response elements are necessary and sufficient for basal and pheromone-induced transcription of the FUS1 gene of Saccharomyces cerevisiae.
AUTHOR: Hagen D C; McCaffrey G; Sprague G F Jr
CORPORATE SOURCE: Institute of Molecular Biology, University of Oregon, Eugene 97403.
CONTRACT NUMBER: GM30027 (NIGMS)
GM07413 (NIGMS)
SOURCE: MOLECULAR AND CELLULAR BIOLOGY, (1991 Jun) 11 (6) 2952-61.
Journal code: NGY. ISSN: 0270-7306.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199109

AB The FUS1 gene of Saccharomyces cerevisiae is transcribed in a and alpha cells, not in a/alpha diploids, and its transcription increases dramatically when haploid cells are exposed to the appropriate mating pheromone. In addition, FUS1 transcription is absolutely dependent on STE4, STE5, STE7, STE11, and STE12, genes thought to encode components of the pheromone response pathway. We now have determined that the pheromone response element (PRE), which occurs in four copies within the FUS1

upstream region, fu[redacted]ons as the FUS1 upstream activ[redacted]on sequence (UAS) and is responsible for all known aspects of FUS1 regu[redacted]tion. In particular, deletion of 55 bp that includes the PREs abolished all transcription, and a 139-bp fragment that includes the PREs conferred FUS1-like expression to a CYC1-lacZ reporter gene. Moreover, three or four copies of a synthetic PRE closely mimicked the activity conferred by the 139-bp fragment, and even a single copy of PRE conferred a trace of activity that was haploid specific and pheromone inducible. In the **FUS1 promoter** context, four copies of the synthetic PRE inserted at the site of the 55-bp deletion restored full FUS1 transcription. Sequences upstream and downstream from the PRE cluster were important for maximal PRE-directed expression but, by themselves, did not have UAS activity. Other yeast genes with PREs, e.g., STE2 and BAR1, are more modestly inducible and have additional UAS elements contributing to the overall activity. In the **FUS1 promoter**, the PREs apparently act alone to confer activity that is highly stimulated by pheromone.

=> s pre-ste?

L21 1387 PRE-STE?

=> s l21 and (fusion? or hybr?)

L22 26 L21 AND (FUSION? OR HYBR?)

=> s l21 (s) (fusion? or hybr?)

L23 22 L21 (S) (FUSION? OR HYBR?)

=> dup rem 123

PROCESSING COMPLETED FOR L23
L24 12 DUP REM L23 (10 DUPLICATES REMOVED)

=> d kwic 1-12

L24 ANSWER 1 OF 12 MEDLINE DUPLICATE 1
AB . . . protein dissociates from G-DNA. Second, MutY removes adenine from oG.A mismatches almost 30-fold faster than from G.A mismatches in a **pre-steady**-state assay, but deletion of the C-terminal domain reduces this specificity for oG.A to less than 4-fold. The kinetic data are consistent with a model in which binding of oG to the C-terminal domain of MutY accelerates the **pre-steady**-state glycosylase reaction by facilitating adenine base flipping. The observation that oG specificity derives almost exclusively from the C-terminal domain of MutY adds credence to the sequence analyses and suggests that specificity for oG.A mismatches was acquired by **fusion** of a MutT-like protein onto the core catalytic domain of an adenine-DNA glycosylase.

L24 ANSWER 2 OF 12 MEDLINE DUPLICATE 2
AB . . . reverse transhydrogenation catalysed by this complex are probably limited by slow release from dIII of NADPH and NADP+, respectively. The **hybrid** complex also catalysed high rates of 'cyclic' transhydrogenation, indicating that hydride transfer, and exchange of nucleotides with dI, are rapid. Stopped-flow experiments revealed a rapid, monoexponential, single-turnover burst of reverse transhydrogenation in **pre-steady**-state. The apparent first-order rate constant of the burst increased with the concentration of rrdI. A deuterium isotope effect (kH/kD approximately. . . al., Eur. J. Biochem. 257 (1998)

202-209), but the differences are readily explained by greater dissociation constant of the **hybrid** complex. The steady state rate of reverse transhydrogenation by the rrdI:hsdIII complex was almost independent of pH, but there was a. . .

L24 ANSWER 3 OF 12 MEDLINE
AB . . . found in Mycobacterium leprae, where TrxR and Trx are encoded by a single gene and, therefore, are expressed as a **fusion** protein (MlTrxR-Trx). This **fusion** enzyme is able to catalyze the reduction of thioredoxin or 5,5'-dithiobis(2-nitrobenzoic acid) or 1, 4-naphthoquinone by NADPH, though the activity. . . the tethered Trx. The reductase has been expressed without Trx attached (MlTrxR). As reported here, comparison of the steady-state and **pre-steady**-state kinetics of MlTrxR-Trx with those of MlTrxR suggests that the low reductase activity of the **fusion** enzyme is an inherent property of the reductase, and that any steric limitation caused by the attached thioredoxin in the **fusion** protein makes only a minor contribution to the low activity. Titration of MlTrxR-Trx and MlTrxR with 3-aminopyridine adenine dinucleotide phosphate. . .

L24 ANSWER 4 OF 12 MEDLINE DUPLICATE 3
AB . . . stem cells are formed or how their identity is initially established. To investigate these questions we have used the P-M **hybrid** dysgenesis syndrome as a tool for ablating the "**pre -stem** cell" progenitors of the stem cells. Our experiments indicate that the **pre-stem** cells in females assume stem cell identity during the early pupal stage. Our results also suggest a model in which at least two **pre-stem** cells must be present within an ovariole for the specification of stem cell fate. When only a single **pre-stem** cell is sequestered by an ovariole, this cell does not assume stem cell identity, but instead follows the cystoblast-cystocyte differentiation. . .

L24 ANSWER 5 OF 12 MEDLINE DUPLICATE 4
AB . . . 34 kDa. We have developed an expression vector that allows the isolation of 40 mg of a glutathione S-transferase (GST)-BVR-A **fusion** protein from 1 litre of culture. The **fusion** protein (60 kDa) behaves as a dimer on gel filtration (120 kDa), so that we have artificially created a BVR-A dimer. The recombinant rat kidney enzyme exhibits **pre-steady**-state 'burst' kinetics that show a pH dependence similar to that already described for ox kidney BVR-A. Similar behaviour was obtained. . .

L24 ANSWER 6 OF 12 MEDLINE DUPLICATE 5
AB . . . two naturally occurring enzyme variants (CATI and CATIII). The introduced charge change greatly facilitates the purification of CATI/CATIII and CATIII/CATIII **hybrid** trimers by ion-exchange chromatography. **Hybrids** containing only one functional active site per trimer were generated in vitro by reversible denaturation of mixtures of "active" subunits (retention of a catalytic histidine at position 195) and "inactive" subunits (with alanine replacing histidine 195). Such **hybrids** were used (1) to demonstrate that the previously observed novel binding of a steroidal antibiotic (fusidic acid) by CATI involves amino acid residues at each subunit interface and (2) to identify specific residues contributing to such interactions. A **pre-steady**-state kinetic characterization of homotrimers containing the H195A substitution also revealed that fusidate binding to CATI may, like chloramphenicol binding, involve. . .

L24 ANSWER 7 OF 12 MEDLINE DUPLICATE 6
AB . . . of the enzyme with a single Cys at position 221 (derived from a gene that was the product of spontaneous **fusion**) showed that this enzyme is still subject to substrate activation [Zeng, X., Farrenkopf, B., Hohmann, S., Jordan, F., Dyda, F.,. . . but not at C222, leads to abolished substrate activation according to a number of kinetic criteria, both steady state and **pre steady** state. On the basis of the three-dimensional structure of the enzyme

[Dyda, F., Furey, W. [redacted]waminathan, S., Sax, M., Farre[redacted]of,. . .

L24 ANSWER 8 OF 12 MEDLINE DUPLICATE 7
AB Mechanistic and structural comparisons of five catalytic monoclonal antibodies generated from the same **hybridoma fusion** indicated that all five hydrolyze phenyl acetate by subtle variations of the same mechanism. All of the antibodies showed a **pre-steady**-state multi-turnover burst in which kcat and Km declined but kcat/Km did not change. The burst of one of the antibodies,. . .

L24 ANSWER 9 OF 12 MEDLINE DUPLICATE 8
AB . . . components, including collagen, contribute significantly to arteriosclerotic changes in the arterial vessel wall. We localized cells actively synthesizing collagen by **hybridizing** 35S-labeled RNA probes complementary to type I and III collagen mRNA with cytoplasmic mRNA in frozen sections of surgically removed. . . model for comparing mRNA levels in areas of high blood pressure-induced wall thickening and in unaffected post-stenotic areas. In situ **hybridization** revealed increased expression of type I and III collagen mRNA in intimal cells and in cells adjacent to the medial-adventitial border in the **pre-stenotic** part of the coarctation. In contrast, cells of the post-stenotic area showed only a very low signal. No immunohistologically detectable macrophages were seen in the **pre-stenotic** subendothelial areas where mRNA levels were enhanced. Higher collagen mRNA levels therefore occur in particular regions of high blood pressure-induced arterial wall thickening in the absence of macrophages. The results suggest that in situ **hybridization** is suitable for detection of locally occurring transcriptional activation of cells for collagens in the vessel wall.

L24 ANSWER 10 OF 12 MEDLINE DUPLICATE 9
AB . . . a few weeks in most cases. The onset age of the shift in preference agreed with the onset age of **fusion**-rivalry discrimination found in a previous study (Birch et al., 1985). The original preference for the bincularly orthogonal patterns may be. . . a grid (interocularly emergent intersections) over a grating, judging from results of two control experiments. These data suggest that the **pre-stereoptic** system non-selectively combines information from the two eyes without regard to edge orientation because it loses eye-of-origin information at a relatively early stage of binocular visual processing. Thus, the **pre-stereoptic** system does not have the capability of interocular suppression. The theoretical and clinical significance of the new findings are discussed. . .

L24 ANSWER 11 OF 12 MEDLINE DUPLICATE 10
AB . . . of irradiated males. Male C57BL/6 mice were irradiated (75-600 rad X-rays to the testes) and were then bred in the **pre-sterile** period to untreated C57BL/6 females. The sperm of their male progeny were examined for the frequency of sperm abnormalities. Variant. . . number of animals studied, similar differences were observed with irradiated male SWR, C3H/He in inbred crosses and with C57BL/6 in **hybrid** crosses with C3H/He females. In contrast, matings of males made at longer times following irradiation did not lead to a. . .

L24 ANSWER 12 OF 12 MEDLINE
AB The culture conditions for the development in vitro of (C57BL/6 x CBA) F2 **hybrid** two-cell embryos to the blastocyst stage have been optimized. Commercially available **pre-sterile** disposable plastic culture dishes supported more reliable development than re-usable washed glass tubes. The presence of an oil layer reduced the variability in development. An average of 85% of blastocysts developed from **hybrid** two-cell embryos cultured in drops of Whitten's medium under oil in plastic culture dishes in an atmosphere of 5% O2:. .

=> s pheremone response element

L25 0 PHEREMONE RESPONSE ELEMENT

=> s pheromone response element

L26 33 PHEROMONE RESPONSE ELEMENT

=> s l26 (s) ste?

L27 21 L26 (S) STE?

=> dup rem l27

PROCESSING COMPLETED FOR L27
L28 11 DUP REM L27 (10 DUPLICATES REMOVED)

=> d kwic 1-11

L28 ANSWER 1 OF 11 MEDLINE DUPLICATE 1
AB . . . been shown to control Tec1 function is the filament response element. We find that the TEC1 promoter also contains several **pheromone response element** sequences which are likely to be functional: TEC1 transcription is induced by mating factor, cell cycle regulated and dependent on the **Ste4**, **Ste18** and **Ste5** components of the mating factor signal transduction pathway. Using alleles of the transcription factor **Ste12** that are defective in DNA binding, transcriptional induction or cooperativity with other transcription factors, we find little correlation between TEC1.
. .

L28 ANSWER 2 OF 11 MEDLINE DUPLICATE 2
AB In the yeast Saccharomyces cerevisiae, **Ste12p** induces transcription of pheromone-responsive genes by binding to a DNA sequence designated the **pheromone response element**. We generated a series of hybrid proteins of **Ste12p** with the DNA-binding and activation domains of the transcriptional activator Gal4p to define a pheromone induction domain of **Ste12p** sufficient to mediate pheromone-induced transcription by these hybrid proteins. A minimal pheromone induction domain, delineated as residues 301 to 335 of **Ste12p**, is dependent on the pheromone mitogen-activated protein (MAP) kinase pathway for induction activity. Mutation of the three serine and threonine. . . potentiate transcription depends on the activity of an adjacent activation domain. These results suggest that the pheromone induction domain of **Ste12p** mediates transcriptional induction via a two-**step** process: the relief of repression and synergistic transcriptional activation with another activation domain.

L28 ANSWER 3 OF 11 MEDLINE DUPLICATE 3
AB The **Ste12p** transcription factor controls the expression of Ty1 transposable element insertion mutations and genes whose products are required for mating in Saccharomyces cerevisiae. The binding site for **Ste12p** is a consensus DNA sequence known as a **pheromone response element** (PRE). Upstream activating sequences (UASs) derived from known **Ste12p**-dependent genes have previously been characterized to require either multiple PREs or a single PRE coupled to a binding site for a second protein. The **Ste12p**-dependent UAS from Ty1, called a **sterile** response element (SRE), is of the second type and is comprised of a PRE and an adjacent TEA (TEF-1, Tec1, and AbaA motif) DNA consensus sequence (TCS). In this report, we show by UV cross-linking analysis that two proteins, **Ste12p** and a protein with an apparent size of 72 kDa, directly contact the Ty1 SRE. Other experiments show that Tec1p. . . the Ty1 SRE and yet another set

of combinatorial int[redacted]ctions that achieve a qualitat[redacted]ly distinct mode of transcriptional regulation with **Ste12p**.

L28 ANSWER 4 OF 11 MEDLINE DUPLICATE 4
AB . . . KAR3 and CIK1 during S/G2 phase was independent of KAR4. A 30-bp region upstream of KAR3 conferred both KAR4- and **STE12**-dependent induction by mating pheromone. This region contained one moderate and two weak matches to the consensus **pheromone response element** to which the **Ste12p** transcriptional activator binds and five repeats of the sequence CAAA(A). Overproduction of **Ste12p** suppressed the kar4 defect in KAR3 induction and nuclear fusion. In contrast, **Ste12p**-independent expression of Kar4p did not alleviate the requirement for **Ste12p** during KAR3 induction. We propose that Kar4p assists **Ste12p** in the pheromone-dependent expression of KAR3 and CIK1. KAR4 defines a novel level of regulation for the pheromone response pathway, acting at a subset of **Ste12p** -inducible genes required for karyogamy.

L28 ANSWER 5 OF 11 MEDLINE DUPLICATE 5
AB . . . insertion mutations of Saccharomyces cerevisiae activate transcription of adjacent genes in a cell-type dependent manner. This activation requires at least **STE12** and TEC1 gene products. The binding site for the **STE12** protein is located in the **sterile** responsive element (SRE), which is just downstream the 5' LTR of Ty1 and contains one copy of the **pheromone response element** (PRE). This report defines the sequences in Ty1 required for TEC1-dependent activation using a TDH3::lacZ reporter gene in which the. . . different portions of a Ty1 or Ty2 element. The Ty1 SRE seems to be sufficient to ensure the TEC1 and **STE12**-mediated activation whereas Ty2 SRE can activate the expression of the adjacent genes in the absence of both proteins. Adjacent to. . . and Ty2 sequences show that Ty1 PAE is required for the activation of adjacent gene expression in a TEC1 and **STE12** -dependent manner. The use of a LexA::TEC1 construct indicates that the chimeric protein has no activation ability suggesting that TEC1 could. . .

L28 ANSWER 6 OF 11 MEDLINE DUPLICATE 6
AB . . . for the basal level of transcription of cell-type-specific genes, as well as the induced level observed after pheromone treatment. The **STE12** protein binds to the DNA sequence designated the **pheromone response element** and is a target of the pheromone-induced signal. We generated 6-nucleotide linker insertion mutants, internal-deletion mutants, and carboxy-terminal truncation mutants of **STE12** and assayed them for their ability to restore mating and transcriptional activity to a **ste12** delta strain. Two of these mutant proteins retain the capacity to mediate basal transcription but show little or no induced. . . the ability to respond to pheromone by increasing gene expression is essential for the mating process. Since distinct domains of **STE12** appear to be required for basal versus induced transcription, we suggest that the pheromone-induced signal is likely to target residues. . . from those targeted by the basal signal because of the constitutive activity of the response pathway. Our analysis of mutant **STE12** proteins also indicates that only the DNA-binding domain is sensitive to the small changes caused by the linker insertions. In addition, we show that, while the carboxy-terminal sequences necessary for **STE12** to form a complex with the transcription factor MCM1 are not essential for mating, these sequences are required for optimal. . .

L28 ANSWER 7 OF 11 MEDLINE DUPLICATE 7
AB **Sterile** mutants of Saccharomyces cerevisiae were isolated from alpha * cells having the a/alpha aar1-6 genotype (exhibiting alpha mating ability and weak a mating ability as a result of a defect in a1-alpha 2 repression). Among these **sterile** mutants, we found two

ste5 mutants together with putative **ste7, ste11**
, and **ste12** mutants of the signal transduction pathway of mating pheromones. The amino acid sequence of the **Ste5p** protein predicted from the nucleotide sequence of a cloned **STE5** DNA has a domain rich in acidic amino acids close to its C terminus, a cysteine-rich sequence, resembling part of. . . and a possible target site of cyclic AMP-dependent protein kinase at its C terminus. Northern (RNA) blot analysis revealed that **STE5** transcription is under a1-alpha 2-Aar1p repression. The MAT alpha 1 cistron has a single copy of the **pheromone response element** in its 5' upstream region, and its basal level of transcription was reduced in these **ste** mutant cells. However, expression of the MAT alpha 1 cistron was not enhanced appreciably by pheromone signals. One of the **ste5** mutant alleles conferred a **sterile** phenotype to a/alpha aar1-6 cells but a mating ability to MATa cells.

L28 ANSWER 8 OF 11 MEDLINE DUPLICATE 8

AB The **STE12** protein of the yeast Saccharomyces cerevisiae binds to the **pheromone response element** (PRE) present in the upstream region of genes whose transcription is induced by pheromone. Using DNase I footprinting assays with bacterially made **STE12** fragments, we localized the DNA-binding domain to 164 amino acids near the amino terminus. Footprinting of oligonucleotide-derived sequences containing one PRE, or two PREs in head-to-tail or tail-to-tail orientation, showed that the N-terminal 215 amino acids of **STE12** has similar binding affinity to either of the dimer sites and a binding affinity 5- to 10-fold lower for the. . . was also evident on a fragment from the MFA2 gene, which encodes the a-factor pheromone. On this fragment, the 215-amino-acid **STE12** fragment protected both a consensus PRE as well as a degenerate PRE containing an additional residue. Mutation of the degenerate. . . The ability of PREs to function as pheromone-inducible upstream activation sequences in yeast correlated with their ability to bind the **STE12** domain in vitro. The sequence of the **STE12** DNA-binding domain contains similarities to the homeodomain, although it is highly diverged from other known examples of this motif. Moreover, the alignment between **STE12** and the homeodomain postulates loops after both the putative helix 1 and helix 2 of the **STE12** sequence.

L28 ANSWER 9 OF 11 MEDLINE

AB . . . increases dramatically when haploid cells are exposed to the appropriate mating pheromone. In addition, FUS1 transcription is absolutely dependent on **STE4, STE5, STE7, STE11,** and **STE12,** genes thought to encode components of the pheromone response pathway. We now have determined that the **pheromone response element** (PRE), which occurs in four copies within the FUS1 upstream region, functions as the FUS1 upstream activation sequence (UAS) and. . . were important for maximal PRE-directed expression but, by themselves, did not have UAS activity. Other yeast genes with PREs, e.g., **STE2** and BAR1, are more modestly inducible and have additional UAS elements contributing to the overall activity. In the FUS1 promoter,. . .

L28 ANSWER 10 OF 11 MEDLINE DUPLICATE 9

AB . . . requirements for conferring both a and alpha mating pheromone inducibilities onto a heterologous promoter. Here we show that the repetitive **pheromone response element** (PRE) which binds to **STE12** protein is sufficient to confer pheromone responsiveness only when present in multiple copies. Moreover, by itself, it is preferentially responsive to alpha factor in a cells. In contrast, a single copy of the PQ box of the **STE3** upstream activation sequence (UAS) is sufficient to confer a-factor responsiveness in alpha cells. The PQ box binds both MCM1 and. . .

L28 ANSWER 11 OF 11 MEDLINE DUPLICATE 10

AB . . . inducible genes, arresting cell division, and forming cell-surface projections. These responses are dependent on the activity of several genes, including **STE12**, whose product binds to the **pheromone response element** located within the regulatory DNA sequences of inducible genes. We assayed the effects of overproducing the **STE12** protein in both **STE**+ cells, as well as **ste2**, **ste7**, and **ste11** mutant cells. We find that overproduction leads to increased transcription of pheromone-inducible genes and is able to suppress the mating defect of the **ste** mutants. These results suggest that one effect of pheromone treatment may be to increase the ability of the **STE12** protein to activate transcription. In addition, we observed that cells cannot tolerate very high levels of the **STE12** protein, and many arrest in G1 with a large size and morphological changes. Thus, constitutively high-level transcription of pheromone-inducible genes. . .

=> d ibib abs 1-2

L28 ANSWER 1 OF 11 MEDLINE DUPLICATE 1
ACCESSION NUMBER: 1998319470 MEDLINE
DOCUMENT NUMBER: 98319470
TITLE: The mating factor response pathway regulates transcription of TEC1, a gene involved in pseudohyphal differentiation of Saccharomyces cerevisiae.
AUTHOR: Oehlen L; Cross F R
CORPORATE SOURCE: The Rockefeller University, New York, NY 10021, USA.
SOURCE: FEBS LETTERS, (1998 Jun 5) 429 (1) 83-8.
Journal code: EUH. ISSN: 0014-5793.
PUB. COUNTRY: Netherlands
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals; Cancer Journals
ENTRY MONTH: 199809
ENTRY WEEK: 19980904
AB The transcription factor Tec1 is involved in pseudohyphal differentiation and agar-invasive growth of Saccharomyces cerevisiae cells. The sole element in the TEC1 promoter that has thus far been shown to control Tec1 function is the filament response element. We find that the TEC1 promoter also contains several **pheromone response element** sequences which are likely to be functional: TEC1 transcription is induced by mating factor, cell cycle regulated and dependent on the **Ste4**, **Ste18** and **Ste5** components of the mating factor signal transduction pathway. Using alleles of the transcription factor **Ste12** that are defective in DNA binding, transcriptional induction or cooperativity with other transcription factors, we find little correlation between TEC1 transcript levels and agar-invasive growth.

L28 ANSWER 2 OF 11 MEDLINE DUPLICATE 2
ACCESSION NUMBER: 1998001568 MEDLINE
DOCUMENT NUMBER: 98001568
TITLE: Transcriptional activation upon pheromone stimulation mediated by a small domain of Saccharomyces cerevisiae Ste12p.
AUTHOR: Pi H; Chien C T; Fields S
CORPORATE SOURCE: Department of Molecular Genetics and Microbiology, State University of New York at Stony Brook, 11794, USA.
CONTRACT NUMBER: GM49065 (NIGMS)
SOURCE: MOLECULAR AND CELLULAR BIOLOGY, (1997 Nov) 17 (11) 6410-8.
Journal code: NGY. ISSN: 0270-7306.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English

FILE SEGMENT: Prior Journals
ENTRY MONTH: 199801

AB In the yeast Saccharomyces cerevisiae, **Ste12p** induces transcription of pheromone-responsive genes by binding to a DNA sequence designated the **pheromone response element**. We generated a series of hybrid proteins of **Ste12p** with the DNA-binding and activation domains of the transcriptional activator Gal4p to define a pheromone induction domain of **Ste12p** sufficient to mediate pheromone-induced transcription by these hybrid proteins. A minimal pheromone induction domain, delineated as residues 301 to 335 of **Ste12p**, is dependent on the pheromone mitogen-activated protein (MAP) kinase pathway for induction activity. Mutation of the three serine and threonine residues within the minimal pheromone induction domain did not affect transcriptional induction, indicating that the activity of this domain is not directly regulated by MAP kinase phosphorylation. By contrast, mutation of the two tyrosines or their preceding acidic residues led to a high level of transcriptional activity in the absence of pheromone and consequently to the loss of pheromone induction. This constitutively high activity was not affected by mutations in the MAP kinase cascade, suggesting that the function of the pheromone induction domain is normally repressed in the absence of pheromone. By two-hybrid analysis, this minimal domain interacts with two negative regulators, Dig1p and Dig2p (also designated Rst1p and Rst2p), and the interaction is abolished by mutation of the tyrosines. The pheromone induction domain itself has weak and inducible transcriptional activity, and its ability to potentiate transcription depends on the activity of an adjacent activation domain. These results suggest that the pheromone induction domain of **Ste12p** mediates transcriptional induction via a two-**step** process: the relief of repression and synergistic transcriptional activation with another activation domain.

=> d his

(FILE 'HOME' ENTERED AT 11:39:18 ON 03 MAY 2000)

FILE 'MEDLINE, BIOSIS' ENTERED AT 11:39:28 ON 03 MAY 2000

L1	155	S	FUS1
L2	0	S	FUS1-STE4
L3	0	S	FUS1-STE5
L4	0	S	FUS1 SAME STE4
L5	0	S	FUS1-STE?
L6	1424375	S	STE?
L7	69	S	L6 (S) L1
L8	35	DUP REM	L7 (34 DUPLICATES REMOVED)
L9	22	S	FUS1-LACZ
L10	5	S	L8 (S) (FUSION OR HYBRIDE)
L11	46	S	BAR1
L12	0	S	BAR1-STE?
L13	0	S	FUS1-KSS?
L14	0	S	BAR1-KSS?
L15	392	S	FUS?-STE?
L16	243	S	L15 (S) (FUSION OR HYBR?)
L17	26	S	FUS2
L18	0	S	FUS2-STE?
L19	4	S	FUS1 PROMOTER
L20	2	DUP REM	L19 (2 DUPLICATES REMOVED)
L21	1387	S	PRE-STE?
L22	26	S	L21 AND (FUSION? OR HYBR?)
L23	22	S	L21 (S) (FUSION? OR HYBR?)
L24	12	DUP REM	L23 (10 DUPLICATES REMOVED)
L25	0	S	PHEREMONE RESPONSE ELEMENT
L26	33	S	PHEROMONE RESPONSE ELEMENT
L27	21	S	L26 (S) STE?

L28 11 DUP REM L[redacted] (10 DUPLICATES REMOVED)

=> log y

NPL

QP501 .J7

STIC-ILL

From: Brannock, Michael
Sent: Wednesday, May 03, 2000 12:20 PM
To: STIC-ILL
Subject: 09/378,046

Please provide the following refs:

Blumer et al Trends Biochem. Sci. 19(236-240)1994

Cobb et al J. Biol. Chem. 270(14843-14846)1995

Marshall Cell 80, 179-185, 1995

Waskiewicz et al. Curr. Opin. Cell Biol. 7, 798-805 1995

Lewin, et al., Curr. Opin Cell. Cell Biol. 7, 197-202, 1995

Piomelli et al., J. Lipid Mediators, 6(1-3)(433-443)1993

Migeon et al., J. Biol. Chem 269(46)29146-52, 1994

Castanon et al., Biochem. Biophys. Res. Comm. 198(2)626-31, 1994

Himmler et al J. Receptor Research 13(1-4)79-94, 19994

Thank you,
michael T. Brannock, Ph.D.
Patent Examiner, AU 1646
Crystal Mall One, 10E18
(703) 306-5876

STIC-ILL

From: Brannock, Michael
Sent: Wednesday, May 03, 2000 12:20 PM
To: STIC-ILL
Subject: 09/378,046

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Blumer et al Trends Biochem. Sci. 19(236-240)1994

Cobb et al J. Biol. Chem. 270(14843-14846)1995

Marshall Cell 80, 179-185, 1995

Waskiewicz et al. Curr. Opin. Cell Biol. 7, 798-805 1995

Lewin, et al., Curr. Opin Cell. Cell Biol. 7, 197-202, 1995

Piomelli et al., J. Lipid Mediators, 6(1-3)(433-443)1993

Migeon et al., J. Biol. Chem 269(46)29146-52, 1994

Castanon et al., Biochem. Biophys. Res. Comm. 198(2)626-31, 1994

Himmler et al J. Receptor Research 13(1-4)79-94, 19994

Thank you,

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Cell, Vol. 80, 179–185, January 27, 1995, Copyright © 1995 by Cell Press

Specificity of Receptor Tyrosine Kinase Signaling: Transient versus Sustained Extracellular Signal-Regulated Kinase Activation

Review

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Summary

A number of different intracellular signaling pathways have been shown to be activated by receptor tyrosine kinases. These activation events include the phosphoinositide 3-kinase, 70 kDa S6 kinase, mitogen-activated protein kinase (MAPK), phospholipase C-γ, and the Jak/STAT pathways. The precise role of each of these pathways in cell signaling remains to be resolved, but studies on the differentiation of mammalian PC12 cells in tissue culture and the genetics of cell fate determination in Drosophila and Caenorhabditis suggest that the extracellular signal-regulated kinase (ERK-regulated) MAPK pathway may be sufficient for these cellular responses. Experiments with PC12 cells also suggest that the duration of ERK activation is critical for cell signaling decisions.

Introduction

Receptor tyrosine kinases are involved in signaling both cell proliferation and differentiation (reviewed by Schlessinger and Ullrich 1992). Their role in determining cell differentiation rather than proliferation is especially well exemplified from genetic studies in Caenorhabditis elegans and Drosophila melanogaster, in which signaling from the Let-23, sevenless, and torso receptors determines cell fate in the absence of proliferation (reviewed by Perrimon, 1993; Dickson and Hafen, 1994). For mammalian cells in tissue culture, the stimulation of quiescent fibroblasts into DNA synthesis and the differentiation of the PC12 chromaffin cell line into sympathetic neurones (Greene and Tischler, 1976) have been much-used experimental systems to investigate receptor tyrosine kinase signaling. A central issue in attempting to understand receptor tyrosine kinase signaling is whether different receptors activate different signal transduction pathways and whether there are distinct pathways for differentiation and proliferation. Numerous studies show that, depending on where it is expressed, the same receptor can signal proliferation or differentiation; for example, the fibroblast growth factor receptor signals differentiation in PC12 neuronal cells but in fibroblasts stimulates proliferation. Such observations indicate the critical importance of cell context in understanding signaling.

Extensive work has now elucidated the principles of signal transduction pathways from receptor tyrosine kinases. Following ligand binding, receptor dimerization, and autophosphorylation, Src homology 2 (SH2) domain–containing proteins are recruited to phosphorylated tyrosine residues on the receptor. These SH2 domain–containing proteins include the p85 components of the phosphoinositide 3-kinase (PI3-kinase) pathway; phospholipase C-γ in the protein kinase C pathway; Src family kinases; and p120–GAP, Shc, and Grb2 in the Ras pathway (reviewed by Schlessinger, 1994). In addition, receptor kinases are able to activate the p91STAT pathway (Fu and Zhang, 1993; Silvennoinen et al., 1993; Sadowski et al., 1993). Recruitment to phosphorylated tyrosine residues on receptors leads to activation of the signaling molecule by a variety of mechanisms: tyrosine phosphorylation in the case of phospholipase C-γ and STATs (Sadowski et al., 1993); conformational changes induced by binding of the SH2 domain to phosphotyrosine for PI3-kinases (Backer et al., 1992; Carpenter et al., 1993) and SH-PTP2 tyrosine phosphatase (Lechleider et al., 1993); and translocation to the plasma membrane for stimulation of Ras guanine nucleotide exchange by Sos (Quilliam et al., 1994; Aronheim et al., 1994).

Attempts to Determine Critical Signaling Events

The issue regarding which of these signaling components are needed for cell proliferation or differentiation has been a much-studied area. Numerous attempts have been made to delineate the importance of a particular signaling component or receptor phosphotyrosine by constructing mutant receptors that lack individual tyrosine residues and therefore cannot recruit SH2-containing proteins to those sites. With a few exceptions, e.g., Coughlin et al. (1989), experiments with mutant receptors tend to show that deletion of any one single site does not compromise the stimulation of DNA synthesis. Such results suggest that there are either parallel signal transduction pathways, each of which can signal DNA synthesis, or that there is redundancy in signaling. One example of redundancy is clearly exemplified by the experiments of Valius and Kazlauskas (1993), which showed that either of two tyrosine phosphorylation sites in the platelet-derived growth factor receptor is sufficient for activation of $p21^{Ras}$ and stimulation of DNA synthesis. What is more difficult to conclude from such experiments is whether a known SH2-containing protein recruited to a particular site is essential to signaling since it is always possible to argue that there is another unknown protein recruited to the same site that is the critical signaling intermediate.

A different way to approach the question of critical signaling pathways comes from genetic studies. Strikingly for the Let-23, sevenless, and torso signaling pathways, defects in ligand or receptor can be compensated for by gain-of-function alleles of Ras (Fortini et al., 1992), Raf (Dickson et al., 1992; Han et al., 1994), MEK (Tsuda et al., 1993), or extracellular signal–regulated kinase (ERK) (Brunner et al., 1994b), all of which lie in the same signaling pathway (see below). Such results are perhaps not so surprising with Ras or Raf for which it has been known for some time that oncogenic forms can liberate signal trans-

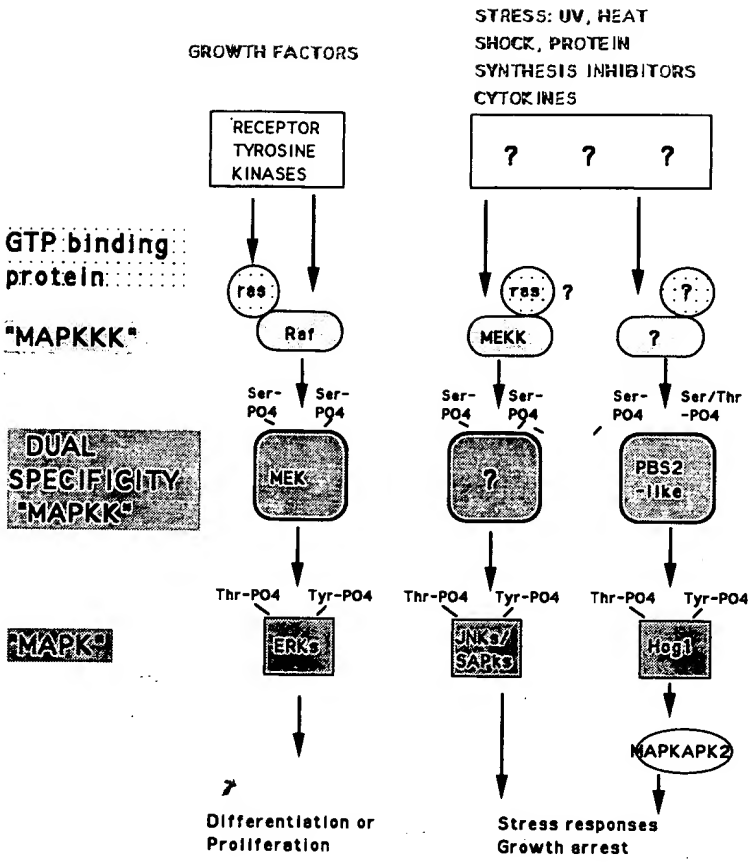

Figure 1. MAPK Pathways in Mammalian Cells

Both the receptor tyrosine kinase pathways and the stress response pathways contain a central core of a serine/threonine kinase, MAPKKK; a dual-specificity kinase, MAPKK; and a serine/threonine kinase, MAPK. Receptor tyrosine kinase signaling ocurrs through $p21^{Ras}$, and there is evidence that some stress responses, e.g., to ultraviolet radiation (UV), may also involve $p21^{Ras}$ (Hibi et al., 1993). Although MEKK-1 activation appears to be Ras-dependent (Lange-Carter and Johnson, 1994), there is no evidence yet for a direct interaction between Ras and MEKK-1.

Note also that not all stimuli leading to Raf activation may be mediated by Ras (Howe et al., 1992; Fabian et al., 1994).

Abbreviations: JNK, Jun kinase; MAPKAPK2, MAPK-activated protein kinase 2; PBS2, a dual-specificity protein kinase in the S. cerevisiae osmotic regulation pathway; PO4, phosphate group; SAPKs, stress-activated protein kinases; and SEK1, appears to be a dual-specificity protein kinase (MAPKK), which activates the JNKs/SAPKs (Sanchez et al., 1994).

duction from extracellular signals. Injection of oncogenic Ras into quiescent fibroblasts stimulates DNA synthesis in the absence of mitogenic growth factors (Ferasmisco et al., 1984; Morris et al., 1989). Oncogenic Ras and Raf will also mimic the effect of nerve growth factor (NGF) in stimulating neurite outgrowth in PC12 cells (Bar-Sagi and Ferasmico, 1985; Noda et al., 1985; Wood et al., 1993). It now appears that Ras may have at least two effectors, Raf and PI3-kinase (Rodriguez-Viciana et al., 1994), and activated Ras would therefore be expected to be able to exert a multiplicity of effects. However, it is more surprising that gain-of-function mutants of MEK and ERK overcome receptor defects since they lie on a single signal transduction pathway downstream of Ras and Raf and would be expected to have more restricted effects. The conclusion that activation of the ERK pathway is sufficient is strengthened by the finding that expression of constitutively activated forms of MEK, generated by site-directed mutagenesis, induces mitogenesis and transformation in fibroblasts as well as differentiation of PC12 cells (Cowley et al., 1994; Mansour et al., 1994).

These results focus attention on the activation of the ERK family of mitogen-activated protein kinases (MAPKs) as a critical event in signal transduction from receptor tyrosine kinases. We will therefore consider some of the aspects of the control of this pathway and a model for how both differentiation and proliferation can be signaled by activation of this pathway.

The Ras/Raf/MEK/ERK Pathway

In the Ras/Raf/MEK/ERK pathway, a small guanine nucleotide-binding protein links receptor tyrosine kinase activation to a cytosolic protein kinase cascade. Central to the activation of this pathway is the activation of Ras to the GTP form through the promotion of guanine nucleotide exchange on Ras. This occurs through the complex of the exchange factor Sos and the adaptor protein Grb2 being recruited to tyrosine-phosphorylated receptors or through Shc–Grb2–Sos complexes (reviewed by Schlessinger, 1994).

The Ras/Raf/MEK/ERK pathway is one example of what are generically termed "MAPK" pathways. MAPK pathways have as their "core" a three-component protein kinase cascade consisting of a serine/threonine protein kinase (MAPKKK), which phosphorylates and activates a dual-specificity protein kinase (MAPKK), which in turn phosphorylates and activates another serine/threonine protein kinase (MAPK) (Figure 1). In the Ras/Raf/MEK/ERK pathway, Raf corresponds to MAPKKK, MEK corresponds to MAPKK, and ERK corresponds to MAPK. These pathways serve to link signals from the cell surface to cytoplasmic and nuclear events. In addition to the receptor tyrosine kinase–coupled Ras/Raf/MEK/ERK pathway, MAPK pathways mediate cell shape, osmotic integrity, and pheromone responses in yeasts (reviewed by Ammerer, 1994; Herskowitz, 1995 [this issue of *Cell*]), stress responses in mammalian cells (Han et al., 1994; Galcheva-

Gargova et al., 1994; Rouse et al., 1994), and cytokine signaling (Freshney et al., 1994) as well as the receptor tyrosine kinase–coupled Ras/Raf/MEK/ERK pathway. It is now emerging that there is a second MAPK pathway in mammalian cells that involves $p21^{Ras}$; this pathway mediates some of the signals that result in the N-terminal phosphorylation of Jun (Figure 1). It involves Ras, MEKK-1, a dual-specificity kinase (SEK1), and the MAPK Jun kinase (JNKs/SAPKs) (Hibi et al., 1993; Minden et al., 1994; Sanchez et al., 1994; Yan et al., 1994).

As far as is known at present from experimental data and sequence comparisons, the mechanisms of activation of the MAPKKs and MAPKs are likely to be identical or very similar in all MAPK pathways. Activation of MEK has been shown to result from phosphorylation by Raf of two serine residues that are four amino acids apart in kinase subdomain VIII (Alessi et al., 1994; Zheng and Guan, 1994). Two identically positioned serine residues or serine and threonine residues within a consensus motif, LID/NSXANS/T, are found in all members of the MAPKK family thus far sequenced (for a recent compilation of MAPKK sequences, see Banuett and Herskowitz, 1994). Substitution of the serine or threonine residues in this consensus with acidic amino acids to mimic phosphorylation leads to partial activation in MEK (Alessi et al., 1994; Mansour et al., 1994). The conservation of the putative phosphorylation sites means that it is very likely that all dual-specificity MAPKKs are regulated in the same way and that their activation can be mimicked by substituting negatively charged amino acids at these sites. All MAPKs contain a TXY motif in kinase subdomain VIII, the phosphorylation of which on threonine and tyrosine is essential for activity.

While identical phosphorylation events are likely to be responsible for the activation of MAPKKs and MAPKs, there appear to be multiple mechanisms for regulating MAPKKKs. For the Schizosaccharomyces pombe pheromone response pathway, there is an obligate requirement for direct interaction between active RasGTP and MAPKKK/byr2 (Van Aelst et al., 1993). However, in the Saccharomyces cerevisiae pheromone MAPK pathways, there does not appear to be a role for Ras in activating MAPKKK/STE11; instead, a kinase STE20 is involved (Leberer et al., 1992). Similarly, in the S. cerevisiae cell wall pathway, the MAPKKK/BCK1 is activated by a protein kinase C homolog, PKC1 (Lee et al., 1993).

The activation of Raf by receptor tyrosine kinases requires $p21^{Ras}$. Recent work demonstrates that the role of Ras is to recruit Raf to the plasma membrane (Leevers et al., 1994; Stokoe et al., 1994), where another tyrosine kinase–generated signal fully activates the membrane-bound Raf (Williams et al., 1992; Leevers et al., 1994; Fabian et al., 1994). Thus, tyrosine kinases generate two signals that interact to activate Raf; one signal is the formation of RasGTP, while the other is unknown. Why two signals should be required to fully activate Raf is a puzzle, but one rationalization is that two signals are used because Ras has multiple effectors. It is now clear that Ras is required for the Raf/Mek/ERK pathway, for PI3-kinase activation (Rodriguez-Viciana et al., 1994), and for a MAPK pathway involving MEKK-1 and the Jun kinases (Lange-Carter and Johnson, 1994; Minden et al., 1994). The second signal would then be needed to acheive specificity, otherwise every signal that activates Ras would activate all effector pathways.

Although there is much still to learn about the roles of each component in MAPK pathways, at the present time it seems that MAPK is essentially the effector of each pathway. The role of MAPKKK may be solely to phosphorylate and activate MAPKK, and the only role of MAPKK is to phosphorylate and activate MAPK. However, MAPK may have multiple substrates and therefore can set in motion a very wide range of events. In the case of the Ras/Raf/MEK/ERK pathway, the substrates of ERK1 and ERK2 include transcription factors and other kinases (reviewed by Blenis, 1993; Treisman, 1994; Hill and Treisman, 1995 [this issue of *Cell*]). Genetic evidence from D. melanogaster strongly suggests that transcription factors are the targets of receptor tyrosine kinase–mediated activation of ERKs. Phosphorylation of these transcription factors may relieve an inhibitory effect in the case of yan (Lai and Rubin, 1992) or lead to activation in the case of pointed (Brunner et al., 1994a).

In PC12 Cells, Cellular Responses Are Determined by the Duration of ERK Activation

The response of PC12 cells to receptor tyrosine kinase activation has been extensively used as an experimental system to study signal transduction and how activation of some receptors leads to differentiation while the activation of others leads to proliferation. Treatment of PC12 cells with fibroblast growth factor or NGF leads to outgrowth of neurites and eventual cessation of cell division (Greene and Tischler, 1976), whereas treatment with epidermal growth factor (EGF) leads to a proliferative signal (Huff et al., 1981). An expectation of these studies was the identification of differentiation-specific pathways. One example has been found in the NGF, but not the EGF, stimulation of tyrosine phosphorylation of an 80 kDa protein, SNT (Rabin et al., 1993). At the qualitative level, many signal transduction events seem to be shared between differentiation and proliferation in PC12 cells (Chao, 1992). However, notable quantitative differences are found between differentiation and proliferative signals. NGF stimulation results in a persistent elevation of RasGTP, whereas EGF produces only a short-lived rise in RasGTP (Muroya et al., 1992). ERK (MAPK) activation is sustained for several hours following NGF stimulation, but it is short lived after EGF stimulation (Heasley and Johnson, 1992; Traverse et al., 1992; Nguyen et al., 1993). In part, these differences may reflect the differences in receptor down-regulation of the EGF receptor and TrkA. Another difference between the two pathways is that EGF and NGF utilize different signal transduction pathways to stimulate RasGTP: the Grb2–Sos complex is directly bound to the EGF receptor, whereas TrkA stimulates Shc phosphorylation and the formation of Shc–Grb2–Sos complexes without direct binding of Grb2–Sos to TrkA (Buday and Downward, 1993; Obermeier et al., 1994; Stephens et al., 1994).

The association of prolonged ERK activation with NGF stimulation of PC12 cells has led to the idea that it is sus-

tained activation of this pathway that leads to differentiation. This is consistent with the findings that both oncogenic Ras and oncogenic Raf can stimulate neurite outgrowth (Noda et al., 1985; Wood et al., 1993), since both of these oncoproteins would be expected to produce prolonged activation of ERKs (Leevers and Marshall, 1992). Two different types of experiments lend support to the idea that there is no receptor-specific pathway of differentiation and that sustained activation of ERKs is sufficient for PC12 differentiation. In the first set of experiments, the effects of heterologous expression of receptors and alterations in endogenous receptor number have been examined. PC12 cells do not express the platelet-derived growth factor receptor, but transfection of platelet-derived growth factor receptor constructs leads to PDGF-dependent differentiation and sustained activation of ERKs (Heasley and Johnson, 1992). The endogenous insulin receptor of PC12 cells does not trigger RasGTP loading, ERK activation, or differentiation (Ohmichi et al., 1993); however, if it is overexpressed, ERK is activated and differentiation results (Dikic et al., 1994). While stimulation of the endogenous EGF receptor does not lead to differentiation, overexpression leads to EGF-dependent differentiation and sustained activation of ERKs (Traverse et al., 1994). Since the EGF receptor is more rapidly downregulated than the NGF receptor through internalization and phosphorylation (Countaway et al., 1992), these results suggest that it is the number of active cell surface receptors that determines whether ERK activation is sustained and differentiation results. An important corollary to receptor overexpression experiments comes from the converse approach. PC12 cell lines have been selected that do not respond to NGF as a differentiation signal. In these cells, the number of TrkA–NGF receptor molecules per cell is reduced, ERK activation is transient, and the response to NGF is proliferation (Schlessinger and Bar-Sagi, 1995). In every situation examined thus far, manipulating receptor numbers to produce differentation correlates with sustained activation of ERKs, whereas transient activation is not associated with differentiation.

The second set of experiments suggesting that ERK activation is the key event in PC12 differentiation is the introduction of MEK mutants. Appropriate mutations in the phosphorylation sites of MEK1 that are the sites of activation by Raf lead to activating and interfering mutants. The interfering mutants block ligand activation of ERK and block differentiation of PC12 cells, whereas the activating mutants induce neurite outgrowth in the absence of differentiating factors (Cowley et al., 1994). The effect of the activated MEK is direct rather than through the induction of growth factors that act back through cell surface receptors because induction of differentiation by activated MEK is not blocked by microinjection of Ras neutralizing antibodies, which block ligand-activated differentiation.

A Model

A model based on these experiments is one that shows cells can enact differentiation or proliferative responses to receptor tyrosine kinases purely on the basis of the duration of ERK activation. An attractive rationalization for

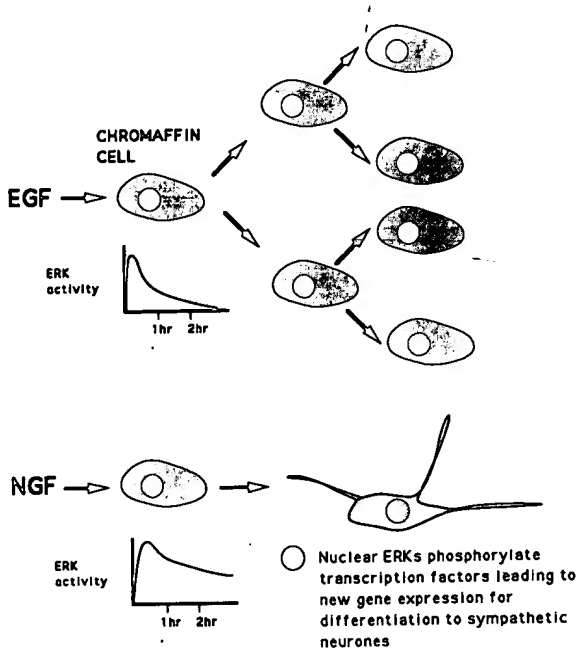

Figure 2. Sustained Activation Leads to Translocation of ERKs and the Induction of New Gene Expression

The relative amount of ERK in the cytoplasm and the nucleus is indicated by the level of stippling.

the manner by which sustained activation of ERKs can lead to a different cellular response than transient activation rests on the observation that ERKs can translocate to the nucleus upon activation (Chen et al., 1992). In every case examined thus far in PC12 cells, sustained ERK activation is associated with translocation of ERKs to the nucleus (Traverse et al., 1992, 1994; Nguyen et al., 1993; Dikic et al., 1994), whereas transient activation does not lead to nuclear translocation. Transient activation will therefore have very different consequences for gene expression compared with sustained activation because nuclear accumulation of active ERK will result in phosphorylation of transcription factors (Figure 2). In this way, the quantitative difference in ERK activation is translated into a qualitative difference in transcription factor activation. Implicit in this model is the idea that the cellular response is determined by which transcription factors are present in the cell. Thus, the activation of the receptor tyrosine kinase and the subsequent activation of ERKs is just the final switch that seals a fate determined by previous developmental events that set which ERK-responsive transcription factors are present in the cell. It is not a requirement of this model that sustained activation of ERKs invariably leads to differentiation, whereas transient activation always leads to proliferation. In other cell types, the converse may be true: it is clear that in fibroblasts, sustained activation of ERKs is associated with proliferation, not differentiation (Meloche et al., 1992; Mansour et al., 1994; Cowley et al., 1994). The important feature of the model is that cells can use transient and sustained activation of ERKs to determine different responses.

The idea of sustained versus transient ERK activation being critical for changes in gene expression can be

readily extended to threshold effects in development, in which small changes in ligand concentration resulting from developmental gradients produce qualitative differences in gene expression (Green et al., 1992). Small differences in ligand concentration could lead to sustained versus transient ERK activation and thereby could lead to nuclear translocation to alter gene expression (Dikic et al., 1994; Traverse et al., 1994).

There are potentially many different ways for receptors to signal transient versus sustained ERK activation. It has already been demonstrated that differences in receptor number markedly affect the duration of ERK activation. As discussed above, small changes in ligand concentration may have similar effects. The rate of internalization of receptors and whether they are down-regulated as a result of activation of the Ras/Raf/MEK/ERK pathway may also affect the duration of signaling. The EGF receptor may be more rapidly internalized than the NGF receptor and is subject to down-regulation through phosphorylation (Countway et al., 1992). In addition, different levels and kinetics of ERK activation could be generated by differential usage of signaling pathways downstream of receptors. The activation of Ras has multiple pathways, including direct receptor–Grb2–Sos complexes (Buday and Downward, 1993), receptor–Syp–Grb2–Sos complexes (Li et al., 1994), and Shc–Grb2–Sos complexes (Obermeier et al., 1994; Stephens et al., 1994). There may also be Ras- (Burgering et al., 1993; Howe et al., 1992) and Raf-independent (Lange-Carter and Johnson, 1994) routes to ERK activation. Each of these different signaling pathways could have different consequences for the level and duration of ERK activation and could be selectively used by different receptors to regulate the amplitude and duration of signaling.

A serious limitation in applying this model to other systems is that it is based wholly on experiments with PC12 cells. It is conceivable that these cells may represent a very abnormal system and that, in other cell types, there are qualitative differences in signaling events for proliferation versus differentiation. However, it is clear from studies in both C. elegans and D. melanogaster that the same components of receptor tyrosine kinase signaling that are involved in the decisions about cell fate are also involved in proliferation since defects in Ras, Raf, MEK, or ERK lead to reduced cell proliferation in embryos and are lethal (Dickson and Hafen, 1994; Tsuda et al., 1993; Perrimon, 1993).

Conclusions

The arguments put forward here propose that when cells make decisions about proliferation versus differentiation through receptor tyrosine kinase signaling, they do it by differences in the duration of ERK activation. Such considerations highlight the importance in development of restricted expression of receptors and especially their ligands (reviewed by Jessell and Melton, 1992). If the presence of growth factors and receptors was not limited in time and space, the activation of a pathway common to all receptor tyrosine kinases would be disastrous. In addition, prior developmental events limit which cells respond to ligand. At one level, this restriction could operate by limiting in which cells a transcription factor is expressed, but restrictions must also operate at other levels. In the D. melanogaster eye, only the R7 cells respond to activation of the sevenless receptor by the bride of sevenless ligand expressed on the R8 cell even though surrounding cells express the receptor and contact the ligand. Exactly where this restriction operates within the Ras/Raf/MEK/ERK pathway is an intriguing issue.

In the model presented here, receptor tyrosine kinases work in development as switches to complete a developmental program determined by previous developmental events. This means that the outcome of receptor tyrosine kinase signaling depends both on the duration of ERK activation and cell context. A critical aspect of cell context will be which ERK-responsive transcription factors are present.

This review has heavily emphasized the role of those receptor tyrosine kinase signaling events that result in ERK activation; the question then remains whether other signaling events contribute to differentiation or proliferation. For example, PI3-kinase activation by recruitment of p85 subunits to either the NGF receptor or the fibroblast growth factor receptor is not necessary for the differentiation response of PC12 cells and does not appear to affect ERK activation (Obermeier et al., 1994; Spivak-Kroizman et al., 1994). Signaling events that do not regulate ERK activation may mediate aspects of tyrosine kinase signaling such as ligand-stimulated cell survival, cytoskeletal rearrangements, cell migration, and chemotaxis (Ridley et al., 1992; Ridley and Hall, 1992). This is illustrated by the finding that activation of protein kinase C by the c-Kit receptor appears to promote cell motility rather than mitogenesis (Blume-Jensen et al., 1993). It is possible that much of the specificity in receptor signaling is for purposes other than controlling proliferation and differentiation.

Acknowledgments

Work in the author's laboratory is supported by the Cancer Research Campaign. I am grateful to Tony Hunter for helpful comments.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 269, No. 46, Issue of November 18, pp. 29146–29152, 1994
Printed in U.S.A.

Regulation of cAMP-mediated Gene Transcription by Wild Type and Mutated G-protein α Subunits

INHIBITION OF ADENYLYL CYCLASE ACTIVITY BY MUSCARINIC RECEPTOR-ACTIVATED AND CONSTITUTIVELY ACTIVATED $G_o\alpha$*

(Received for publication, May 13, 1994, and in revised form, August 29, 1994)

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We have used a luciferase reporter gene under the transcriptional control of a cAMP response element (CRE) to monitor the effects of G-protein α subunits on cAMP-regulated gene expression and to examine muscarinic acetylcholine receptor (mAChR) functional coupling to G-proteins. Expression in JEG-3 cells of a mutationally activated $G_i\alpha$-2 in which glutamine 205 is replaced with leucine (Q205L) decreased forskolin-stimulated expression from the CRE-luciferase gene by up to 75%. Similarly, mutation of glycine 43 (corresponding to glycine 12 in $p21^{ras}$) to valine decreased forskolin-stimulated expression from the CRE-luciferase gene by a maximum of 50%, indicating that this mutation activates the G-protein and is potentially oncogenic. Transfection of the activated Q205L $G_o\alpha$ subunit decreased forskolin stimulation of CRE-luciferase expression. Transfected wild type $G_o\alpha$ was also able to couple the m4 mAChR receptor to inhibition of AC. The amino-terminal myristoylation site was removed from wild type $G_i\alpha$-2 and Q205L $G_i\alpha$-2 by changing glycine 2 to alanine (G2A). $G_i\alpha$-2 with the G2A and Q205L mutations was unable to decrease forskolin stimulation of CRE-mediated luciferase activity. Furthermore, G2A $G_i\alpha$-2 was unable to couple the m4 mAChR to inhibition of AC. Thus, myristoylation is required both for the function of constitutively active Q205L $G_i\alpha$-2 and for receptor-mediated activation of wild type $G_i\alpha$-2.

Guanine nucleotide binding regulatory proteins (G-proteins)[1] are heterotrimers consisting of α, β, and γ subunits. The α subunit is unique for each G-protein and contains the site of GTP binding and hydrolysis as well as sites for receptor and effector interaction. This subunit can be a substrate for ADP-ribosylation by bacterial toxins, and some α subunits contain sites for the attachment of fatty acids such as myristic acid. Several forms of β and γ subunits have also been identified. Their function is less well understood, but they are involved in direct signaling and possibly also in membrane localization. There are several classes of G-proteins, defined by their α subunits. The best characterized are G_s and G_i, which, while named for their abilities to stimulate and inhibit adenylyl cyclase (AC) can also interact with various ion channels. The functional coupling of some of these G-proteins has been determined; G_o can regulate the activity of K^+ and Ca^{2+} ion channels, G_q couples to stimulation of phospholipase C, and G_s can inhibit AC. Individual classes of G-proteins are capable of coupling to multiple effectors; for example, G_i can activate both phospholipase C and K^+ ion channels in addition to inhibition of AC activity (see Simon *et al.* (1991) and Spiegel *et al.* (1992) for review).

Various mutations have been identified that constitutively activate the diverse G-protein α subunits. Activation is often due to inhibition of the intrinsic GTPase activity. Furthermore, the oncogenes *gsp* and *gip*2 have been shown to result from mutations in G_s and G_i, respectively (Landis *et al.*, 1989; Lyons *et al.*, 1990). To examine the function of various wild type and mutated G-proteins α subunits, we have used a luciferase reporter gene under the transcriptional control of a cAMP response element (CRE) as a sensitive monitor of intracellular cAMP levels and cAMP-regulated gene expression. In JEG-3 cells transfected with the CRE-luciferase construct, forskolin activation of AC resulted in a 7–10-fold increase in luciferase activity. This system has several advantages: one can detect small but physiologically relevant changes in intracellular cAMP levels and there is no background signal from untransfected cells. Furthermore, one can perform transient transfections to assay the functional consequences of diverse combinations of cDNAs encoding proteins involved in signal transduction. We have used this system to examine the function and coupling of wild type and mutated G-protein α subunits.

EXPERIMENTAL PROCEDURES

DNA and Expression Vectors—The chick m4 mAChR genomic clone (Tietje *et al.*, 1990), the rat $G_i\alpha$-2 (Jones and Reed, 1987), the rat Q205L $G_i\alpha$-2 (Hermouet *et al.*, 1991), the rat $G_o\alpha$ (Jones and Reed, 1987), the rat Q205L $G_o\alpha$ (Wong *et al.*, 1992), the rat G2A $G_i\alpha$-2, and the rat G2A,Q205L $G_i\alpha$-2 G-protein α subunit cDNAs were expressed in the expression vector pCD-PS (Bonner *et al.*, 1988). The myristoylation deficient G-proteins G2A $G_i\alpha$-2 and G2A,Q205L $G_i\alpha$-2 were generated by using polymerase chain reaction to introduce the mutation glycine 2 to alanine (Saiki *et al.*, 1985). To ensure that the correct mutations were generated and that no other mutations were introduced, the fragments of DNA generated by polymerase chain reaction were sequenced (Sanger *et al.*, 1977) using Sequenase (United States Biochemical Corp.). The human $G_i\alpha$-2 (Beals *et al.*, 1987) and the G43V $G_i\alpha$-2 (Beals, 1989) G-protein α subunit cDNAs were expressed in the expression vector pNUT (Palmiter *et al.*, 1987). The α-inhibin CRE-luciferase plasmid consisted of a CRE containing 74 bp *Bst*XI (−170) to *Nco*I (−96) fragment from the α-inhibin promoter (Pei *et al.*, 1991) blunt end ligated into the TK-105 luciferase plasmid. The constitutively active RSV-β-galactoside construct (Edlund *et al.*, 1985) has been described previously (Day *et al.*, 1989).

Cell Transfection and Culture—JEG-3 cells were obtained from the America Type Culture Collection and were grown in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum. Transient transfection of JEG-3 cells and subsequent assays

* This research was supported by National Institutes of Health grants (to N. M. M.) and by training grants GM07108, GM07750, and NS07332. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: G-protein, guanine nucleotide-binding regulatory protein; AC, adenylyl cyclase; CRE, cAMP response element; mAChR, muscarinic acetylcholine receptor; RSV, Rous sarcoma virus.

of luciferase and β-galactosidase activity were carried out as described by Mellon *et al.* (1989). Cells seeded at 2.5×10^4 cells/15-mm well were transiently transfected with between 200 and 300 ng of total DNA by calcium phosphate precipitation 72 h after plating. The transfection mixes consisted of 15 ng/well of the α-inhibin CRE-luciferase gene construct, 40 ng/well of RSV-β-galactosidase to normalize for transfection efficiency, and 100 ng/well of receptor or G-protein plasmids. Carrier DNA was used to ensure that all transfections within a given experiment have a constant amount of total DNA. The medium was replaced 24 h after transfection and cells were treated with the appropriate drug another 24 h later. Triplicate wells were treated with various drugs for 5 h before harvesting of cells.

Immunoblot Analysis of G-protein Expression—Immunoblot analysis of G-protein expression were carried out as described by Luetje *et al.* (1987). Two 60–80% confluent 100-mm plates of JEG-3 cells were transiently transfected with 10 μg of total DNA by calcium phosphate precipitation. Forty-eight hours after transient transfection, cells were washed with ice-cold phosphate-buffered saline (20 mM NaH_2PO_4, 150 mM NaCl, pH 7.4). Cells were scraped and dounce homogenized in phosphate-buffered saline containing protease inhibitors (0.4 mM phenylmethylsulfonyl fluoride, 1 mM 1,10-phenanthroline, 1 mM iodoacetamide, 1 μM pepstatin A). Nuclei were removed by centrifugation at $1000 \times g$ for 5 min. Membranes were separated from the cytosol by centrifugation at $150,000 \times g$ for 40 min. Samples from the membrane and cytosolic fractions were analyzed by SDS-polyacrylamide gel electrophoresis. Partially purified G_i and G_o, prepared as described by Milligan and Klee (1985), was obtained from Dr. A. M. Spiegel. Immunoblot analysis was performed as described previously (Luetje *et al.*, 1987) using antisera AS7 (Goldsmith *et al.*, 1987) and alkaline phosphatase-conjugated IgG for detection of $G_i\alpha$-2, Q205L $G_i\alpha$-2, G43V $G_i\alpha$-2, G2A $G_i\alpha$-2, and G2A,Q205L $G_i\alpha$-2.

Assay of Luciferase Activity—After removal of media, transfected cells were harvested by solubilization in 100 μl of extraction buffer (100 mM KPO_4, 4 mM ATP, 1.5 mM $MgSO_4$, 1 mM dithiothreitol, 0.1% Triton X-100). 25 μl of cell extract was added to 350 μl of luciferase assay buffer (100 mM KPO_4, 4 mM ATP, 1.5 mM $MgSO_4$) in luminometer cuvettes. The luminometer injects 100 μl of D-luciferin (1 mM; Analytical Luminescence Laboratories, Inc.) into each sample and determines luminescence over 30 s. Luciferase counts were divided by β-galactosidase values (see below) to determine normalized luciferase activity.

Assay of β-Galactosidase Activity—25 μl of cell extract were added to microtiter plate wells containing 200 μl of β-galactosidase assay buffer (60 mM $NaPO_4$, 10 mM KCl, 1 mM $MgCl_2$, 50 mM β-mercaptoethanol). 40 μl of the β-galactosidase substrate o-nitrophenyl-β-galactopyranoside (2 mg/ml; Calbiochem) was added to each well and plates were colorimetrically assayed for β-galactosidase activity.

RESULTS

Expression of Wild Type and Mutated $G_i\alpha$-2 Constructs in Transfected JEG-3 Cells—Previous studies using both Northern blot and immunoblot analysis have reported that JEG-3 cells express $G_i\alpha$-1 and $G_i\alpha$-3 but not $G_i\alpha$-2 or $G_o\alpha$ (Montmayeur *et al.*, 1993). JEG-3 cells were transfected with the various $G_i\alpha$-2 constructs resulting in the transient expression of the corresponding proteins when cells were harvested 48 h later. The transfected proteins were identified on immunoblots of the soluble and particulate fractions of homogenized cells. Using the antiserum AS7 which is specific to $G_i\alpha$-1 and $G_i\alpha$-2 with weak cross reactivity to $G_i\alpha$-3, we were able to visualize protein bands at approximately 40 kDa. Expression of wild type rat and human $G_i\alpha$-2, the glutamine 205 to leucine $G_i\alpha$-2 (Q205L), and the glycine 43 to valine $G_i\alpha$-2 (G43V) was easily detectable above background in the particulate fractions of the lysed and homogenized cells (Fig. 1*A*). There was no detectable expression of these subunits in the soluble fraction of the transfected cells. Expression of the $G_i\alpha$-2 constructs in which the amino-terminal myristoylation site has been removed by changing glycine 2 to alanine (G2A) was detectable in the soluble fractions of cell homogenates (Fig. 1*B*). This is consistent with previous observations that myristoylation is necessary for membrane attachment of inhibitory G-proteins (Jones *et al.*, 1990; Mumby *et al.*, 1990). We did however detect significant amounts of the myristoylation mutants in the membrane fractions. Gallego *et al.* (1992) also observed this in immunoblots of these same constructs, and the nonmyristoylated yeast GPA1 α subunit can also be found in the membrane fraction (Stone *et al.*, 1991). While expression of G2A $G_i\alpha$-2 in the membrane fraction appears to be less than that of the wild-type $G_i\alpha$-2, the total expression of G2A $G_i\alpha$-2 from both the membrane and cytosolic fractions is equal to or greater than the total expression of the wild type $G_i\alpha$-2. Furthermore, immunoblots of transiently transfected COS-7 cells also showed that the expression levels of wild type and mutant G2A $G_i\alpha$-2 were identical (data not shown).

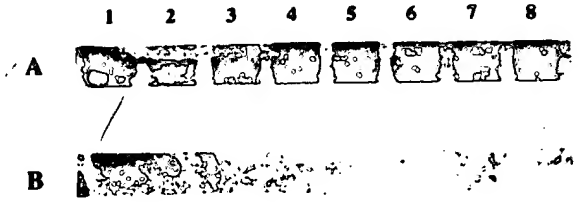

FIG. 1. **Expression of wild type and mutated $G_i\alpha$2 constructs in transiently transfected JEG-3 cells.** Immunoblot analyses of G-protein expression were carried out as described by Luetje *et al.* (1987). Membranes and cytosolic fractions were prepared from transiently tranfected JEG-3 cells and subjected to SDS-polyacrylamide gel electrophoresis. The antisera AS7 was used for detection of human and rat wild type $G_i\alpha$-2, rat Q205L $G_i\alpha$-2, human G43V $G_i\alpha$-2, rat G2A $G_i\alpha$-2, and rat G2A,Q205L $G_i\alpha$-2. *A*, membrane protein (25 μg/lane). *Lanes*: *1*, purified G_i/G_o; *2*, control JEG-3; *3*, human wild type $G_i\alpha$-2; *4*, human G43V $G_i\alpha$-2; *5*, rat wild type $G_i\alpha$-2; *6*, rat Q205L $G_i\alpha$-2; *7*, rat G2A $G_i\alpha$-2; *8*, rat G2A,Q205L $G_i\alpha$-2. *B*, soluble proteins (60 μg/lane). *Lanes*: *1*, purified G_i/G_o; *2*, control JEG-3; *3*, human wild type $G_i\alpha$-2; *4*, human G43V $G_i\alpha$-2; *5*, rat wild type $G_i\alpha$-2; *6*, rat Q205L $G_i\alpha$-2; *7*, rat G2A $G_i\alpha$-2; *8*, rat G2A,Q205L $G_i\alpha$-2. While expression of the human G43V $G_i\alpha$-2 in this blot appears to be greater than that of wild type human $G_i\alpha$-2, in two other experiments, expression of wild type human $G_i\alpha$-2 was equal to or greater than expression of G43V $G_i\alpha$-2.

Transient Expression of the Activated Q205L $G_i\alpha$-2 in JEG-3 Cells Attenuates Forskolin Stimulation of CRE-mediated Luciferase Expression—To test the feasibility of using the CRE-luciferase assay to analyze functional mutations in G-protein α subunits, we compared the effects of wild type and constitutively activated $G_i\alpha$ subunits. Transfection of JEG-3 cells with a cDNA expression construct encoding the oncogenic Q205L $G_i\alpha$-2 mutation decreases forskolin-stimulated expression from the CRE-mediated luciferase gene as compared with control DNA or wild type $G_i\alpha$-2 (Fig. 2). At low concentrations of forskolin, stimulation of CRE-mediated luciferase expression was inhibited by up to 75%. These data are consistent with previous experiments demonstrating constitutive activation of $G_i\alpha$-2 by this mutation and demonstrate that the CRE-luciferase expression system can be used to examine the effects of potential activating G-protein mutations on intracellular cAMP levels.

Transient Expression of G43V $G_i\alpha$-2 in JEG-3 Cells also Attenuates Forskolin Stimulation of CRE-mediated Luciferase Expression—We next tested whether mutation of glycine 43 to valine also activates the $G_i\alpha$-2 G-protein subunit. This mutation corresponds to the p21ras activating mutation in which glycine 12 is changed to valine. Studies by different groups of the effects of the analogous mutation (G49V) in $G_s\alpha$ have yielded inconsistent conclusions (see "Discussion"), and the effects of this mutation in $G_i\alpha$-2 have not been previously reported. Transfection with a cDNA expression construct encoding G43V $G_i\alpha$-2 also depresses forskolin-stimulated CRE-luciferase expression up to a maximum of 50% at low concentrations of forskolin (Fig. 3). Thus, the glycine 43 to valine mutation also activates this G-protein.

Transient Expression of the Activated Q205L $G_o\alpha$ in JEG-3 Cells Attenuates Forskolin Stimulation of CRE-mediated Luciferase Expression—Transfection of activated Q205L $G_o\alpha$ has

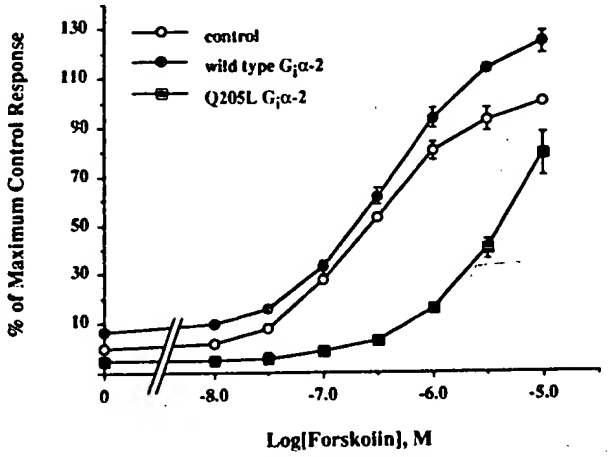

FIG. 2. **Transient expression of activated Q205L $G_i\alpha$2 in JEG-3 cells attenuates forskolin stimulation of CRE-mediated luciferase expression.** Control DNA (*open circle*), wild type $G_i\alpha$-2 (*filled circle*), and Q205L $G_i\alpha$2 (*filled square*) expression vectors (100 ng/well) were cotransfected with the α-inhibin luciferase reporter gene (15 ng/well) and the RSV β-galactosidase gene (40 ng/well). Transfected cells were treated with increasing concentrations of forskolin. Data is plotted as the percentage of the maximum control response and values are mean ± S.E., $n = 3$.

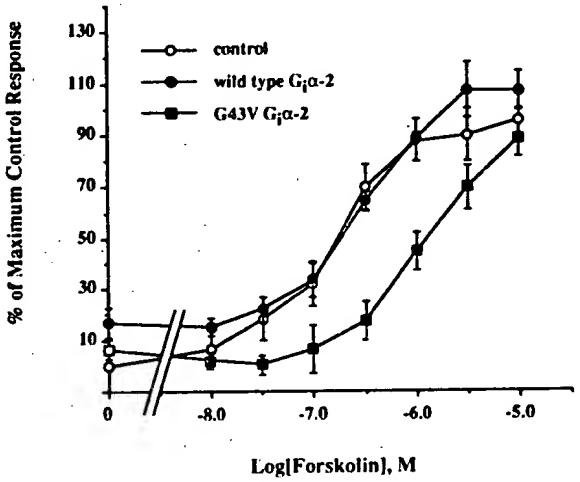

FIG. 3. **Transient expression of the activated G43V $G_i\alpha$2 in JEG-3 cells attenuates forskolin stimulation of CRE-mediated luciferase expression.** Control DNA (open circle), wild type $G_i\alpha$-2 (filled circle), and G43V $G_i\alpha$2 (filled square) expression vectors (100 ng/well) were cotransfected with the α-inhibin luciferase reporter gene (15 ng/well) and the RSV β-galactosidase gene (40 ng/well). Transfected cells were treated with varying concentrations of forskolin. Data is plotted as the percentage of the maximum control response and values are mean ± S.E., $n = 3$.

been reported to have no effect on either basal or forskolin-stimulated levels of intracellular cAMP (Wong *et al.*, 1992). In contrast, reconstitution of purified $G_o\alpha$ into either pertussis toxin-treated neuroblastoma cell membranes or into Sf9 cell membranes expressing type I AC has been reported to reconstitute inhibition of AC (Carter and Medzihradsky, 1993; Taussig *et al.*, 1994). Because the CRE-luciferase assay provides a sensitive measure of intracellular cAMP concentration in transiently transfected cells, we tested the ability of $G_o\alpha$ to mediate inhibition of AC in intact cells. Transfection of a cDNA expression construct encoding Q205L $G_o\alpha$ subunit decreased forskolin-stimulated expression from the CRE-mediated luciferase gene as compared with control DNA or wild type $G_o\alpha$ (Fig. 4).

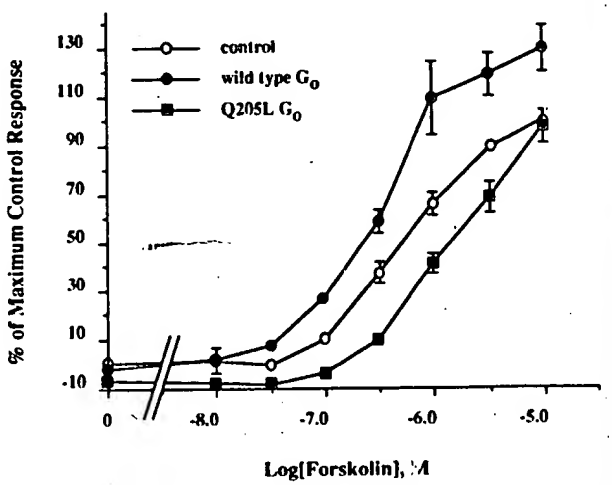

FIG. 4. **Transient expression of the Q205L $G_o\alpha$ in JEG-3 cells attenuates forskolin stimulation of CRE-mediated luciferase expression.** Control DNA (*open circle*), wild type $G_o\alpha$ (*filled circle*), and Q205L $G_o\alpha$ (*filled square*) expression vectors (100 ng/well) were cotransfected with the α-inhibin luciferase reporter gene (15 ng/well) and the RSV β-galactosidase gene (40 ng/well). Transfected cells were treated with varying concentrations of forskolin. Data are plotted as the percentage of the maximum control response and values are mean ± S.E., $n = 3$.

Maximal inhibition occurred at low concentrations of forskolin and stimulation of CRE-mediated luciferase expression was inhibited by up to 50%. Thus, the activated Q205L $G_o\alpha$ can mediate inhibition of AC in intact JEG-3 cells.

$G_o\alpha$ Can Couple the m4 mAChR to Inhibition of AC—We have previously demonstrated that m4 receptors preferentially couple to $G_i\alpha$-2 over $G_i\alpha$-1 and $G_i\alpha$-3 to inhibit forskolin-stimulated AC activity (Migeon and Nathanson, 1994). To further test the ability of $G_o\alpha$ to couple to inhibition of AC, cDNAs encoding the m4 mAChR and $G_o\alpha$ were cotransfected into JEG-3 to determine whether $G_o\alpha$ could also couple the m4 receptor to inhibition of AC. Transfected cells were treated with 0.316 μM forskolin and varying concentrations of carbachol. $G_o\alpha$, like $G_i\alpha$-2 and unlike $G_i\alpha$-1 and $G_i\alpha$-3 (Migeon and Nathanson, 1994), was able to reconstitute m4 mAChR inhibition of forskolin-stimulated luciferase activity (Fig. 5). Thus, $G_o\alpha$ can mediate m4 mAChR inhibition of AC in JEG-3 cells.

$G_o\alpha$ Mediation of m4 mAChR Inhibition of CRE-Luciferase Expression Is Not Due to $G_o\alpha$-mediated Decreases in Intracellular Ca^{2+} Levels—Previous work (Migeon and Nathanson, 1994) has demonstrated that the expression of the CRE-luciferase reporter gene in JEG-3 cells is relatively insensitive to changes in intracellular Ca^{2+} levels; treatment with Ca^{2+} ionophore increased luciferase expression by 1.5–2-fold, in contrast to 7–12 fold increases with forskolin. It has been suggested that transfected $G_o\alpha$ can inhibit isoproterenol-induced cAMP accumulation in C6 glioma cells by inhibition of Ca^{2+} influx and thus decreasing intracellular Ca^{2+} levels (Charpentier *et al.*, 1993). We therefore tested whether treatment of JEG-3 cells with drugs that would increase intracellular Ca^{2+} would have an effect on $G_o\alpha$-mediated m4 mAChR inhibition of forskolin-stimulated CRE-luciferase expression. While treatment with A23187 and ionomycin slightly increased basal levels of CRE-luciferase expression, there was no effect on $G_o\alpha$-mediated m4 inhibition of forskolin-stimulated luciferase activity (Fig. 6). Thus, $G_o\alpha$-mediation of cAMP accumulation is not due to $G_o\alpha$ inhibition of a Ca^{2+} channel.

Myristoylation Is Required for Function of the Q205L $G_i\alpha$-2 in JEG-3 Cells—In order to test whether myristoylation is necessary for function of the activated Q205L $G_i\alpha$-2, we removed the

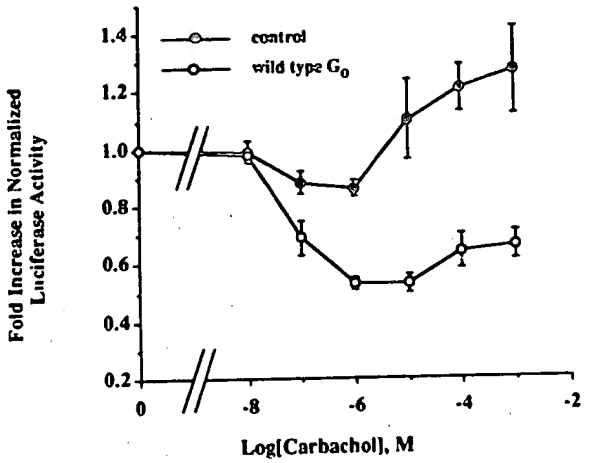

FIG. 5. **m4 couples to $G_o\alpha$ for inhibition of forskolin-stimulated AC activity.** Control (*open circle*) and $G_o\alpha$ (*filled circle*) expression vectors 100 ng/well were cotransfected with m4 expression vector (10 ng/well), α-inhibin luciferase reporter gene (15 ng/well) and the RSV β-galactosidase gene (40 ng/well). Transfected cells were treated with 0.316 μM forskolin and varying concentrations of carbachol. Data are shown as -fold increases in normalized luciferase activity and values are mean ± S.E., $n = 3$.

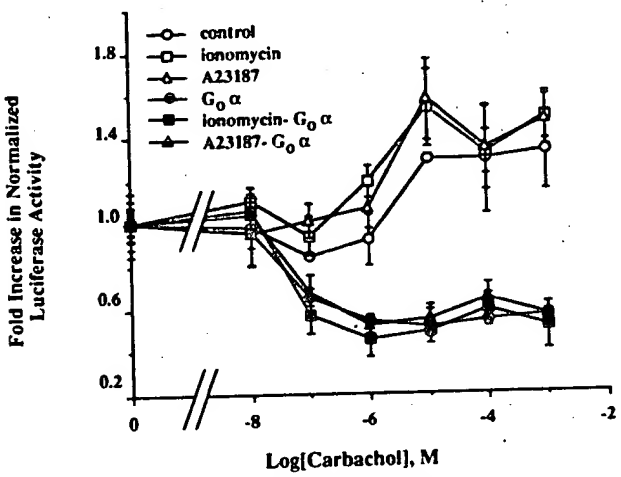

FIG. 6. **$G_o\alpha$-mediated m4 inhibition of forskolin-stimulated AC activity is insensitive to increases in intracellular Ca^{2+}.** Control (*open symbols*) and $G_o\alpha$ (*filled symbols*) expression vectors (100 ng/well) were cotransfected with m4 expression vector (10 ng/well), α-inhibin luciferase reporter gene (15 ng/well) and the RSV β-galactosidase gene (40 ng/well). Transfected cells were treated with control (*circles*) media containing 0.316 μM forskolin, 0.5 mM $CaCl_2$ (in addition to the Ca^{2+} already in the media), and varying concentrations of carbachol. Ionomycin (1 μM) (*squares*) and A23187 (1 μM) (*triangles*) were added to the above treatment mixes. Data are shown as fold increases in normalized luciferase activity and a representative experiment is shown. Values are means of triplicate determinations ± S.D. Two other experiments yielded similar results. Treatment with ionomycin and A23187 did result in a 1.5–2-fold stimulation of basal luciferase/β-galactosidase values from 618 to 938 and 1225, respectively, for the control transfections, and from 1064 to 1578 and 1483, respectively, for the $G_o\alpha$ transfections. This was consistent with previous observations of the relative Ca^{2+} insensitivity of the AC isoforms expressed in JEG-3 cells, but did not alter the ability of $G_o\alpha$ to mediate m4 inhibition.

site of myristoylation by also changing glycine 2 to alanine (G2A). Whereas Q205L depresses forskolin-stimulated luciferase expression, transfection of JEG-3 cells with the G2A,Q205L $G_i\alpha$-2 does not do so (Fig. 7). Thus, myristoylation is required for the function of the activated G-protein.

Myristoylation Is Required for $G_i\alpha$-2 Coupling of m4 mAChR to Inhibition of Adenylyl Cyclase—While the results in Fig. 7

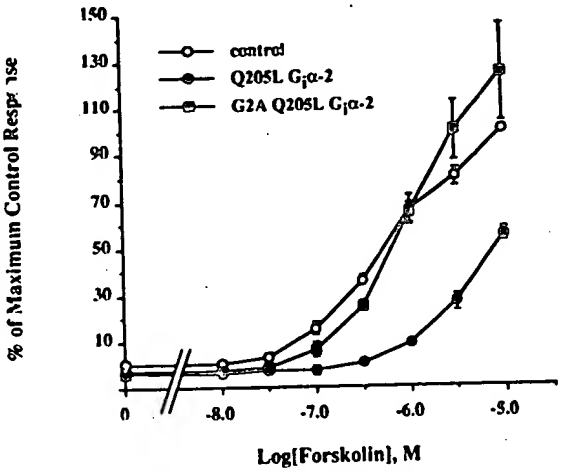

FIG. 7. **Transient expression of the G2A,Q205L $G_i\alpha$2 in JEG-3 cells does not attenuate forskolin stimulation of CRE-mediated luciferase expression.** Control DNA (*open circle*), $G_i\alpha$-2 Q205L (*filled circle*), and G2A $G_i\alpha$-2 Q205L (*filled square*) expression vectors (100 ng/well) were cotransfected with the α-inhibin luciferase reporter gene (15 ng/well) and the RSV β-galactosidase gene (40 ng/well). Transfected cells were treated with varying concentrations of forskolin. Data are plotted as the percentage of the maximum control response and values are mean ± S.E., $n = 3$.

demonstrate that myristoylation is required for the function of a constitutively activated mutated $G_i\alpha$-2, they do not indicate whether myristoylation is required for receptor-mediated signal transduction. To further test the role of myristoylation in receptor-mediated G-protein function, we also removed the myristoylation site from wild type $G_i\alpha$-2. Previously we have shown that in JEG-3 cells, m4 mAChR preferentially couples to $G_i\alpha$-2 to inhibit forskolin-stimulation of luciferase activity. As JEG-3 cells lack endogenous $G_i\alpha$-2, co-transfection of $G_i\alpha$-2 is required for optimal m4-mediated inhibition of forskolin-stimulated CRE-luciferase expression (Migeon and Nathanson, 1994). In cotransfection experiments, the G2A $G_i\alpha$-2 no longer couples m4 mAChR to inhibition of AC (Fig. 8). Thus, myristoylation is necessary for coupling $G_i\alpha$-2 to receptor-mediated signal transduction.

DISCUSSION

The importance of characterizing mutations that activate G-proteins is clear as such mutations have been detected in human tumors. Activating mutations in $G_s\alpha$ have been found in human pituitary tumors (Landis *et al.*, 1989; Clementi *et al.*, 1990) and similar mutations in $G_i\alpha$-2 have been found in human adrenal and ovarian tumors (Lyons *et al.*, 1990). The G_s and $G_i\alpha$ oncogenes have been named *gsp* and *gip*2, respectively. The oncogenic potentials of activating G-protein mutations have been examined in various cell lines; R179C and Q205L $G_i\alpha$-2 have been shown to be transforming in Rat 1 cells (Pace *et al.*, 1991; Gupta *et al.*, 1992). While activated Q205L $G_o\alpha$ was able to transform NIH 3T3 cells (Kroll *et al.*, 1992), expression of Q205L $G_i\alpha$-2 in that same cell line was not transforming but did stimulate proliferation (Gupta *et al.*, 1992). The precise role of G-proteins in mitogenesis remains unknown, but it is clear that all the subfamilies are involved in varying degrees and in a cell type specific manner.

The JEG-3 cell CRE-luciferase system is a useful tool for studying the effects of transfected wild type and mutated G-protein subunits on intracellular cAMP levels. Measurement of cAMP-regulated gene expression is a sensitive way to detect subtle but physiologically relevant changes in intracellular cAMP levels that might not be possible using direct biochemical

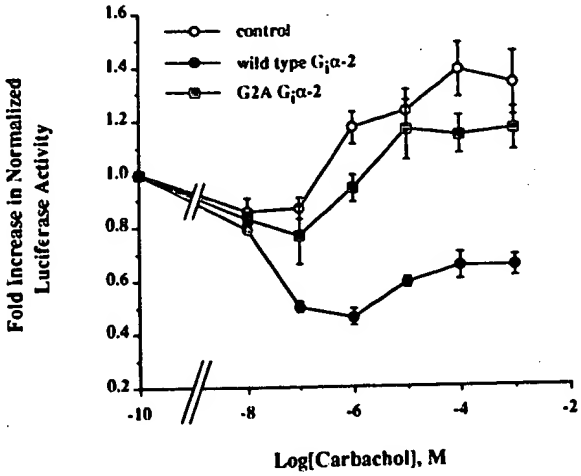

FIG. 8. **G2A $G_i\alpha$-2 is unable to couple the m4 mAChR to inhibition of forskolin-stimulated AC activity.** Control (*open circle*), wild type $G_i\alpha$-2 (*filled circle*), and G2A $G_i\alpha$-2 (*filled square*) expression vectors (100 ng/well) were cotransfected with m4 expression vector (10 ng/well), α-inhibin luciferase reporter gene (15 ng/well) and RSV β-galactosidase gene (40 ng/well). Transfected cells were treated with 0.316 μM forskolin and varying concentrations of carbachol. Data is shown as fold increases in normalized luciferase activity and values are means ± S.E., $n = 3$.

measurements of cAMP. This system has been used to characterize the functional consequences of mutations in genes for cAMP-dependent protein kinase (Mellon *et al.*, 1989). We originally adapted this system to study muscarinic acetylcholine receptor-mediated effects on intracellular cAMP levels (Migeon and Nathanson, 1994). We show here that this system can be used to determine the effects of wild type and mutated G-proteins on intracellular cAMP levels. To establish the suitability of this system for studying activating G-protein mutations, we transfected JEG-3 cells with DNA encoding the well characterized activated Q205L $G_i\alpha$-2. This mutation in p21ras (Q61L) (Gibbs *et al.*, 1985) is transforming and the corresponding mutation in the $G_s\alpha$ subunit (Q227L) inhibits the intrinsic GTPase activity (Masters *et al.*, 1989; Graziano and Gilman, 1989). Transfection with Q205L $G_i\alpha$-2 clearly inhibits forskolin-stimulated increases in CRE-luciferase expression and is consistent with the findings of other laboratories (Hermouet *et al.*, 1991; Wong *et al.*, 1991) using stable transfections and direct biochemical determinations of intracellular cAMP levels.

Having demonstrated the effectiveness of these techniques in detecting activating G-protein mutations, we determined the functional status of G43V $G_i\alpha$-2. As in the case of the Q205L mutation, the corresponding G43V mutation in $p21^{ras}$ (G12V) is transforming. However, the effect of the corresponding mutation on the function of the $G_s\alpha$ subunit (G49V) is less clear. G49V $G_s\alpha$ causes a small elevation of basal intracellular cAMP levels and basal protein kinase A activity when stably expressed in Chinese hamster ovary cells (Woon *et al.*, 1989) and shows increased activity when reconstituted with bovine brain AC (Graziano and Gilman, 1989). The intrinsic GTPase activity of G49V $G_s\alpha$ was also inhibited as compared to wild type $G_s\alpha$ (Masters *et al.*, 1989; Graziano and Gilman, 1989). However, while these data suggests that G49V $G_s\alpha$ is activated *in vitro*, when G49V $G_s\alpha$ was stably expressed in S49 cyc^- cells, a $G_s\alpha$ deficient mouse lymphoma cell line, AC activation by GTP and other activators was less than what was seen with cells expressing wild type $G_s\alpha$ (Masters *et al.*, 1989). Also, while transient expression of G49V $G_s\alpha$ in COS-7 cells activated AC, stable expression of the mutated G_s in PC12 cells inhibited AC (Kabir *et al.*, 1993). Thus, the functional status of the G49V $G_s\alpha$ remains ambiguous. The G43V mutation in $G_i\alpha$-2 has not previously been characterized. We demonstrate here that transfection of G43V $G_i\alpha$-2 into JEG-3 cells inhibited forskolin stimulation of CRE-mediated luciferase activity.

The Q205L and G43V mutations maximally inhibited CRE-luciferase activity by 75% and 50% respectively. Because the expression levels of Q205L $G_i\alpha$-2 and G43V $G_i\alpha$2 are comparable, G43V $G_i\alpha$-2 appears to possess less constitutive activity than Q205L $G_i\alpha$-2. The differing activities of the two $G_i\alpha$-2 mutations in JEG-3 cells is also manifested in the activities of the corresponding mutations in the $G_s\alpha$ subunit: Masters *et al.* (1989) found that Q227L $G_s\alpha$ was a better activator of AC than G49V (Masters *et al.*, 1989), and the relative activities of the mutated $G_s\alpha$ subunits were found to correlate with the degree of their inhibition of the wild type GTPase activity, 95% and 45% respectively (Graziano and Gilman, 1989).

Transfection of JEG-3 cells with Q205L $G_o\alpha$ also attenuates forskolin-stimulated luciferase activity and wild type $G_o\alpha$ can couple the m4 mAChR to inhibition of AC. These findings indicate that in JEG-3 cells, $G_o\alpha$ can regulate AC. There have been a variety of conflicting reports on the ability of $G_o\alpha$ to inhibit AC activity. Expression of Q205L $G_o\alpha$ in HEK 293 and NIH 3T3 cells did not attenuate either basal or forskolin-stimulated levels of intracellular cAMP (Wong *et al.*, 1992). In contrast to these experiments in intact cells, addition of G_o has recently been shown to reconstitute coupling of the μ opiate receptor to inhibition of AC in pertussis-toxin treated membranes from brain and the neural-derived cell line SH-SY5Y (Carter and Medzihradsky, 1993). Similarly, in experiments in which membranes derived from Sf9 cells expressing various AC isoforms are reconstituted with purified G-protein α subunits, GTPγS activated $G_o\alpha$ inhibited type I AC and not types II, V, and VI (Taussig *et al.*, 1994). The work presented here clearly demonstrates that $G_o\alpha$ can inhibit AC activity in response to both constitutively activating mutations and mAChR activation. Thus, our data are consistent with the observations of other groups studying AC activity in membranes. Interestingly, we believe that, in our experiments, $G_o\alpha$ is not inhibiting the Ca^{2+}-calmodulin-sensitive type I AC because CRE-luciferase expression in JEG-3 cells is relatively insensitive to increases in intracellular Ca^{2+}.

Muscarinic receptor functional coupling to $G_o\alpha$ has been suggested by *in vitro* reconstitution studies in which chick cardiac m2 and m4 receptors were shown to activate G_o (Richardson *et al.*, 1991), and activation of reconstituted recombinant m2 receptors stimulates the binding of GTPγS to a variety of G-protein α subunits including G_o (Tota *et al.* 1990; Parker *et al.*, 1991). The only direct evidence of mAChR functional coupling through G_o is in GH_3 cells where mAChRs mediate inhibition of a Ca^{2+} current. Injection of GH_3 cells with antisense oligonucleotides against the G_{o1} form of $G_o\alpha$ abolishes mAChR-mediated inhibition of a Ca^{2+} current (Kleuss *et al.*, 1991). In this work we demonstrate m4 mAChR coupling to $G_o\alpha$ to inhibit AC.

There have also been reports of indirect regulation of AC by $G_o\alpha$; expression of $G_o\alpha$ in C6 glioma cells partially inhibits β-adrenergic receptor stimulated accumulation of cAMP by inhibiting a transient Ca^{2+} influx that is required for 50% of the increases in cAMP (Charpentier *et al.*, 1993). We do not believe that this is the mechanism of mAChR-mediated inhibition of CRE-luciferase expression in JEG-3 cells because CRE-luciferase expression in JEG-3 cells is relatively insensitive to changes in intracellular Ca^{2+} and treatment with drugs that increase intracellular Ca^{2+} had no significant effect on $G_o\alpha$-mediated m4 inhibition of CRE-luciferase expression.

Although G-proteins α subunits generally behave like integral membrane proteins, there is no indication that they contain any transmembrane segments. These observations have prompted interest in the the mechanism of G-protein membrane localization (Spiegel *et al.*, 1991). One hypothesis is that the βγ subunits complex is anchored in the membrane and the the α subunit is localized to the membrane by association with the βγ complex. In support of this hypothesis it has been shown that α subunits do not associate with artificial phospholipid vesicles in the absence of βγ subunits (Sternweis, 1986). If βγ subunit complexes anchor the α subunits, then one might expect to see α subunits released from the membrane when they dissociate from the βγ complex upon G-protein activation. α subunits can be released by treatment of membranes with detergent or high pH (Audigier *et al.*, 1990), but, with G-protein activation, release of α subunits occurs very slowly if at all (Buss *et al.*, 1987; Milligan *et al.*, 1988). Overexpression of G-protein α subunits in excess of βγ subunits has no effect on membrane localization (Simonds *et al.*, 1989). Furthermore, α subunits expressed in yeast lacking βγ subunits are still membrane localized (Blumer and Thorner, 1991).

Another hypothesis is that lipid modification of G-protein subunits is responsible for G-protein membrane attachment. While G-protein α subunits have been shown to be palmitoylated in the amino-terminal region (Linder *et al.*, 1993), the significance of this is not yet well understood. G_o and $G_i\alpha$ subunits have been shown to be myristoylated on an amino-terminal glycine residue (Buss *et al.*, 1987; Schultz *et al.*, 1987; Jones *et al.*, 1990). When the amino-terminal glycine is mutated to alanine, thus blocking myristoylation, the mutated α subunits are found mostly in the cytosol (Jones *et al.* 1990; Mumby *et al.*, 1990). Myristoylation of the $G_o\alpha$ subunit also increases its affinity for βγ subunits (Linder *et al.*, 1991). We have made the G2A mutation in both wild type and Q205L $G_i\alpha$-2 and have found that myristoylation is necessary for function of both the wild type and the mutated G-proteins. G2A,Q205L $G_i\alpha$-2 does not behave like Q205L $G_i\alpha$-2 and has little effect on forskolin-stimulated luciferase activity. These observations are consistent with other work (Gallego *et al.*, 1992) which showed that the G2A,Q205L $G_i\alpha$-2 was unable to inhibit basal or cholera toxin-stimulated intracellular cAMP. They also showed that myristoylation of Q205L $G_i\alpha$-2 was required for transformation of Rat 1a cells and stimulation of MAP kinase activity. More importantly, we have shown that the G2A $G_i\alpha$-2 can no longer couple the m4 mAChR to inhibition of forskolin-stimulated CRE-luciferase expresssion. This is the first demonstration that myristoylation is necessary for receptor-mediated activation of the $G_i\alpha$-2 subunit in intact cells.

Thus, we have shown that the JEG cell CRE-luciferase reporter system is useful for studying the effects of various G-protein mutations and receptor-G-protein coupling. We have shown that the G43V $G_i\alpha$-2 is activated and that G_o can mediate inhibition of AC. Furthermore, G_o was shown to mediate m4 inhibition of forskolin-stimulated luciferase activity. We also examined the role of myristoylation in the function of activated and wild type $G_i\alpha$-2 subunits and found that myristoylation was required for their function. While we concentrated on the G_i and G_o α subunits, one might also use this system to look at the function of other G-proteins involved in regulation of intracellular cAMP levels. For example, analysis of structure-function relationships for $G_s\alpha$ and $G_o\alpha$ should be amenable to this type of assay system. Similarly, the function of G-proteins α subunits which activate protein kinase C (by activation of phospholipase C) might be analyzed by using a luciferase reporter gene construct driven by an AP-1-regulated promoter element instead of the CRE-luciferase reporter gene construct.

Acknowledgments—We are grateful to Dr. Randy Reed (Johns Hopkins University) for the gift of the rat $G_i\alpha$2 G-protein α subunit cDNA, Drs. Roger Perlmutter and Chan Beals (University of Washington) for the gift of the human $G_i\alpha$2 and $G_i\alpha$2 G43V cDNAs, Dr. Kelly Mayo (Northwestern University) for the gift of the α-inhibin CRE-luciferase reporter gene construct, Dr. Henry Bourne (University of California at San Francisco) for the gift of rat G_o Q205L, Dr. Tom Bonner (National Institute of Mental Health) for the gift of pCD-PS, and Dr. Allen Spiegel for the gifts of rat $G_i\alpha$2 Q205L, purified G/G_o, and the AS7 antiserum. We would also like to thank Dr. Stan McKnight for his kind help and advice.

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Article title	Dopamine D_2 receptor signaling via the arachidonic acid cascade: Modulation by cAMP-dependent protein kinase A and prostaglandin E_2
Article identifier	092183199300051G
Authors	Piomelli_D Di_Marzo_V
Journal title	Journal of Lipid Mediators
ISSN	0921-8319
Publisher	Elsevier USA
Year of publication	1992
Volume	6
Issue	1-3
Supplement	0
Page range	433-443
Number of pages	11
User name	Adonis
Cost centre	Development
PCC	$20.00
Date and time	Wednesday, May 03, 2000 2:57:38 PM

Journal of Lipid Mediators, 6 (1993) 433–443

LIPMED 00216 8.3

Dopamine D_2 receptor signaling via the arachidonic acid cascade: modulation by cAMP-dependent protein kinase A and prostaglandin E_2

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Summary

Recent studies have shown that, in Chinese hamster ovary cells transfected with D_2-receptor cDNA, CHO(D_2) cells, D_2 agonists are potent in enhancing the release of [^{3}H]arachidonic acid (AA) induced by stimulation of constitutive purinergic receptors or by application of Ca^{2+} ionophores. This facilitatory action is further amplified by the concomitant activation of D_1 receptors, which per se have no effect on evoked [^{3}H]AA release. Here, we review a series of experiments aimed at examining the molecular mechanism of this synergistic interaction. The results show that, in CHO(D_2) cells: (a) application of 8-Br-cAMP or stimulation of constitutive prostaglandin (PG)E_2 receptors augment the AA response produced by D_2 agonists; (b) in CHO(D_2) cells transfected with human β_2-receptor cDNA, the β-agonist, isoproterenol, produces a similar effect; (c) the potentiation of [^{3}H]AA release produced by PGE_2 and 8-Br-cAMP is prevented by overexpressing either a protein inhibitor of cAMP-dependent protein kinase (PKA) or a mutated form of pKA regulatory subunit incable of binding cAMP; (d) mock-synergism is obtained in CHO(D_2) cells overexpressing the catalytic subunit of PKA; (e) PGE_2 is a major AA metabolite in stimulated CHO(D_2) cells and its formation may contribute to the effect of D_2 agonists on AA release. The results indicate

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that cAMP-induced activation of PKA represents a likely molecular basis for D_1/D_2 receptor synergism on AA release. They also suggest that additional membrane receptors, colocalized with D_2 and positively linked to adenylyl cyclase, may exert a similar action. Furthermore, stimulation of PGE_2 receptors by endogenously produced prostaglandin may participate in AA signaling at the D_2 receptor, by providing a paracrine positive feedback loop.

Key words: Chinese hamster ovary cells; cyclic AMP; cAMP-dependent protein kinase A; Dopamine receptors; Phospholipase A_2; Prostaglandin $(PG)E_2$; Transfected cells

1. Introduction

Along with classical second messengers, which include such diverse molecules as cyclic nucleotides, inositol trisphosphate and diacylglycerol, arachidonic acid (AA) released through G protein-mediated activation of phospholipase A_2 (PLA_2), has recently been shown to participate in intracellular signaling at several neurotransmitter receptors in the CNS (Piomelli et al., 1987; Dumuis et al., 1990; Felder et al., 1990). A characteristic of the AA signaling cascade is its ability to give rise to a large array of bioactive metabolites, which can act either as intracellular second messengers, by modulating the activity of ion channels and protein kinases, or, if released from the cell of origin, as paracrine mediators, by binding to G protein-coupled membrane receptors on neighboring cells (for review, see Wolfe and Coceani, 1979; Axelrod et al., 1988; Piomelli and Greengard, 1990; Shimizu and Wolfe, 1990).

Dopamine, a major modulatory neurotransmitter, exerts its diverse actions in the brain by binding to two functional classes of G protein-coupled membrane receptors: 'D_1-like' receptors (which include the recently cloned D_{1A}, D_{1B} and D_5 subtypes) are positively linked to adenylyl cyclase and phospholipase C activities, whereas 'D_2-like' receptors (which include D_{2A}, D_{2B}, D_3 and D_4) either inhibit or exert no effect on the activities of these enzymes (reviewed by Sibley and Monsma, 1992, and Vallar and Meldolesi, 1989). Despite these opposing actions on classical second messenger systems, D_1-like and D_2-like receptors can act synergistically at the level of single neurons, to produce many electrophysiological and behavioural responses (Clark and White, 1987; Bertorello et al. 1990; Calabresi et al., 1992). This suggests that an additional, unknown second messenger pathway activated by dopamine receptors may underlie their synergistic interactions.

We and others have recently reported that, in Chinese hamster ovary cells transfected with D_2-receptor cDNA, $CHO(D_2)$, D_2 agonists facilitate the release of AA induced by stimulation of Ca^{2+}-mobilizing receptors (Kanterman et al., 1991; Felder et al., 1991; Piomelli et al., 1991). This facilitation is specific for D_2 receptors (both D_{2A} and D_{2B}): stimulation of CHO cells transfected with either D_1- or D_3-receptor cDNA has no effect on the levels of evoked AA release

(Piomelli et al., 1991). However, in cells coexpressing D_1 and D_2, occupation of both receptor subtypes results in a marked synergistic potention of release (Piomelli et al., 1991). The results suggest that amplification of the AA cascade may represent a possible molecular basis for the synergism between D_1 and D_2 receptors observed in the brain at the electrophysiological and behavioral level.

In this study, we describe a series of experiments aimed at examining the molecular mechanism underlying D_1/D_2-receptor synergism on AA release. Because D_1 receptors enhance cAMP levels in transfected CHO cells even when D_2 receptors are stimulated concomitantly (Piomelli et al., 1991), we investigated the possible involvement of cAMP and cAMP-dependent protein kinase A (PKA) in the synergistic interaction on AA. Using combined pharmacological and molecular genetic approaches, we show here that PKA participates in regulating AA signal-

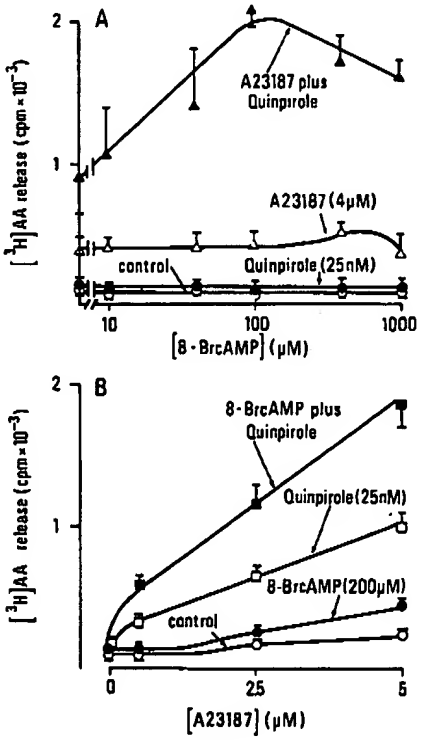

Fig. 1. Actions of 8-bromo-cAMP on D_2 receptor-dependent potentiation of [^{3}H]AA release in CHO(D_2) cells. (A) Concentration-dependent responses to 8-bromo-cAMP applied alone, or in the presence of quinpirole (25 nM0, A23187 (4 μM) or quinpirole plus A23187. (B) Effects of 8-bromo-cAMP on the release of [^{3}H]AA evoked by A23187 (0.5–5 μM), or by A23187 plus quinpirole (25 nM0. CHO(D_2) cells, labeled by incubation with [^{3}H]AA (0.25 μCi/ml) for 2 h; were incubated for 30 min in 1 ml of Dulbecco's modified Minimal Essential Medium (DMEM) containing 0.1% bovine serum albumin (BSA) and final concentrations of the appropriate drugs (Piomelli et al., 1991). Samples (0.5 ml) of the incubation media were collected for liquid scintillation counting. Points represent the mean ± SE of 4–8 separate determinations.

ing at the D_2 receptor. In addition, we report evidence indicating that dopaminergic activation evokes release of prostaglandin (PG)E_2 from CHO(D_2) cells. Because PGE_2 enhances, in turn, the AA response produced by D_2-receptor occupation, this prostaglandin may act as a paracrine messenger to modulate dopaminergic responses (Di Marzo and Piomelli, 1992).

2. cAMP Enhances the Potentiation of AA Release Produced by Stimulating D_2 Receptors

Three different sets of experiments were designed to examine a possible participation of intracellular cAMP in D_1/D_2-receptor synergism on AA release. First, we studied the effects of 8-Br-cAMP, a stable cAMP analogue, on the AA response to quinpirole, a D_2 receptor agonist. When CHO(D_2) cells were stimulated with a combination of Ca^{2+} ionophore, A23187 (2 μM), quinpirole (25 nM) plus 8-Br-cAMP, release of [^{3}H]AA was much greater than in cells stimulated with A23187 plus quinpirole alone. By contrast, 8-Br-cAMP had little or no direct effect on A23187-evoked [^{3}H]AA release (Fig. 1).

Next, we evaluated the effect of stimulating constitutive PGE receptors, which are coupled to adenylyl cyclase activation. Incubation of CHO(D_2) cells with either PGE_1 or PGE_2, but not with $PGF_{2\alpha}$, produced significant accumulation of intracellular cAMP (from 0.4 ± 0.06 to 1.0 ± 0.06 pmol/well with 0.2 μM PGE_1) and enhanced the effect of quinpirole (0.2 μM) on [^{3}H]AA release (Fig. 2). With PGE_1, maximal stimulation of [^{3}H]AA release was at 1 μM prostaglandin ($221 \pm 20\%$ of quinpirole), and half-maximal effect was obtained at a concentration (EC_{50}) of 140 nM. With PGE_2, maximal stimulation was $146 \pm 8\%$ at 1 μM

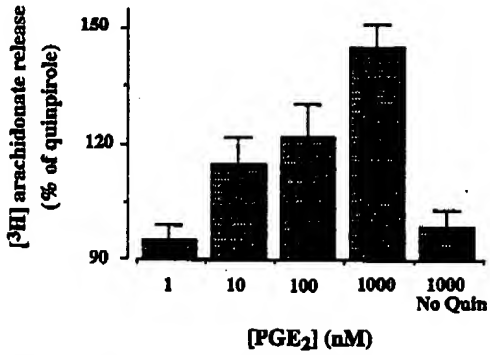

Fig. 2. Stimulation of constitutive PGE_2 receptors enhances D_2 receptor-dependent potentiation of [^{3}H]AA release, CHO(D_2) cells were labeled with [^{3}H]AA and stimulated as described in the legend to Fig. 1. Results are expressed as percentage of the release produced by A23187 plus quinpirole (956 ± 144 cpm per well) and are the mean $\pm$ SE (bars) of 5 experiments carried out in quadruplicate. The effect of all but 1 nM concentration of PGE_2 were significantly different ($P < 0.05$) from both the effect of A23187 plus quinpirole and the effect of A23187 plus PGE_2. (Reproduced, with permission, from the Journal of Neurochemistry.)

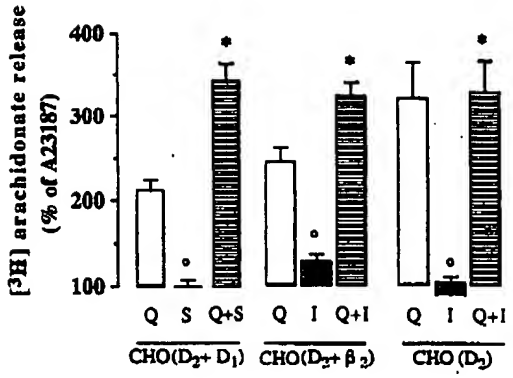

Fig. 3. Stimulation of transfected β_2 receptors enhances D_2 receptor-dependent potentiation of [^{3}H]AA release. CHO(D_2) cells were stably transfected with a cDNA encoding for the human β_2 receptor by using the lipofectin method and a phleomycin-resistance gene as marker. Clones expressing functional β_2 receptors were selected on the basis of their ability to increase cAMP levels in response to applications of isoproterenol, a β_2 agonist. Isoproterenol had no effect on cAMP levels in untransfected CHO(D_2) cells. Transfection of CHO(D_2) cells with the D_1 receptor cDNA was carried out as described (Piomelli et al., 1991). Cell labeling and stimulations were as described in the legend to Fig. 1. Q, quinpirole (0.25 μM); I, isoproterenol (1 μM); S, compound SKF-38393, a D_1 receptor agonist (1 μM).

prostaglandin and EC_{50} was 100 nM. Little or no direct effect of either PGE_1 or PGE_2 was seen on A23187-evoked [^{3}H]AA release in the absence of quinpirole (Fig. 2).

In order to substantiate further the hypothesis that receptor-dependent stimulation of adenylyl cyclase may act synergistically with D_2 to potentiate AA release, CHO(D_2) cells were permanently transfected with a cDNA encoding for the human β_2-adrenergic receptor. CHO($D_2 + \beta_2$) cells were stimulated with isoprotenerol, a β_2 agonist, in the presence of ionophore (4 μM) and quinpirole (1 μM). Isoprotenerol had little or no effect on [^{3}H]AA release in the absence of D_2 receptor activation, but it significantly enhanced the potentiating action of quinpirole (Fig. 3). This effect of isoproterenol was prevented by the β_2-adrenergic antagonist, propranolol, and enhanced by incubation with the phosphodiesterase inhibitor, isobutylmethylxanthine (data not shown).

3. cAMP-dependent Protein Kinase Participates in Regulating AA Signaling at the D_2 Receptor

The results, showing that raises in intracellular cAMP levels potentiate D_2 agonist-induced enhancement of [^{3}H]AA release, suggest that cAMP-dependent protein kinase (PKA) may be involved in mediating this response. To examine this possibility we adopted two experimental strategies: by using a molecular genetic approach, we attempted either to reduce the activity of endogenous PKA or to enhance it by overexpressing exogenous PKA in CHO(D_2) cells.

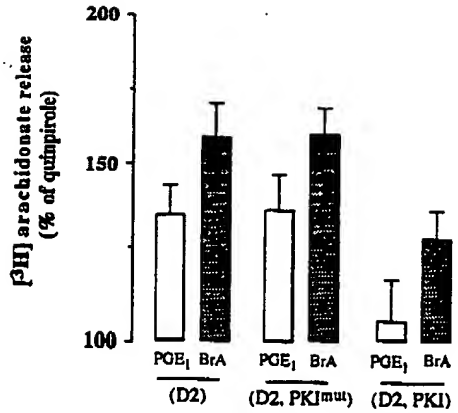

Fig. 4. Overexpression of the PKA inhibitory protein, PKI, prevents cAMP-dependent enhancement of the D_2 AA response. CHO(D_2) cells were transiently transfected with a cDNA encoding for rat PKI or for a mutated inactive form of PKI (PKI^{mut}) (Day et al., 1989) by using the lipofectin method. Cell labeling and stimulations were as described in Fig. 1. Experiments were carried out 36–48 h after transfections. Results are the mean ± SE (bars) of 3 separate transfections, carried out in quadruplicate. BrA, 8-bromo-cAMP (100 μM).

To reduce endogenous PKA activity, we transiently transfected CHO(D_2) cells with vectors directing expression of either of two PKA inhibitory proteins: the heat-stable PKA inhibitor (PKI) (Day et al., 1989), or a PKA regulatory subunit mutated in both the A and B cAMP-binding sites (MTR-EV_{AB}) (Mellon et al., 1989). The effect of PKI overexpression on [^{3}H]AA release from CHO(D_2) cells is shown in Fig. 4. In CHO(D_2 + PKI) cells, the potentiating actions of either PGE_1 (1 μM) or 8-bromo-cAMP (100 μM) were greatly reduced when compared to untransfected cells or to cells that had been transfected with a mutated, inactive form of PKI (Fig. 4). Next, to obtain overexpression of mutated PKA regulatory subunit, we used a vector designed to direct expression of this protein under the control of the Mt-1 promoter, a moderately strong promoter that can be further induced by incubating cells in a medium containing Zn^{2+} (Mellon et al., 1989). Exposure to Zn^{2+} (80 μM, 36 h) did not affect the AA response of untransfected CHO(D_2) cells to the application of 8-bromo-cAMP. However, in MTR-EV_{AB}-transfected CHO(D_2) cells exposed to Zn^{2+}, the potentiation of [^{3}H]AA release caused by the cAMP analog was markedly inhibited (Fig. 5). Similar results were obtained when cells were stimulated with PGE_1 (not shown).

To construct CHO(D_2) cells expressing unregulated PKA activity, we transfected the cells transiently with an Mt-1-linked vector, directing expression of the mouse catalytic subunit of PKA (MTC-EVα) (Mellon et al., 1989). After 36 h of culture in a Zn^{2+}-containing medium, the response to the D_2 agonist, quinpirole, was markedly stronger in MTC-EVα-transfected than in control cells (Fig. 6).

The experiments with CHO(D_2) cells deficient or enriched in PKA activity, described above, indicate that this protein kinase participates in regulating the

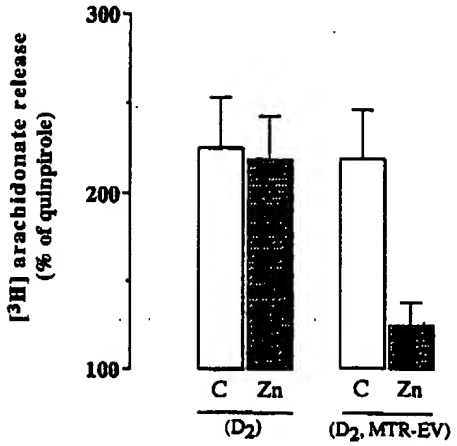

Fig. 5. Overexpression of a mutated regulatory subunit of PKA, which inhibits PKA activity, prevents 8-bromo cAMP-dependent enhancement of the D_2 AA response. CHO(D_2) cells were transiently transfected with the cDNA encoding for mouse PKA RII regulatory subunit, mutated on both A and B cAMP-binding sites (MTR-EV_{AB}) (Mellon et al., 1990), by using the lipofectin method. Cell labeling and stimulations were as described in Fig. 1. Cells were cultured for 36 h either without (C), or with 80 μM Zn^{2+} (Zn) in the culture medium, and stimulated with A23187 (2 μM), quinpirole (0.1 μM) and 8-bromo cAMP (100 μM), as described in Fig. 1. Data are the mean$\pm$SE (bars) of 4 separate determinations from one transfection.

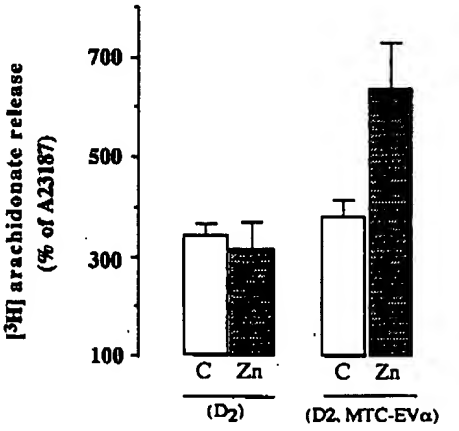

Fig. 6. Overexpression of the catalytic subunit of PKA amplifies D_2 receptor-dependent potentiation of [^{3}H]AA release. CHO(D_2) cells were transiently transfected with a cDNA encoding for mouse PKA catalytic subunit (MTC-EV_α) (mellon et al., 1990) by using the lipofectin method. Cell labeling and stimulations were as described in Fig. 1. Cells were cultured for 36 h either without (C), or with 80 μM Zn^{2+} (Zn) in the culture medium, and stimulated with A23187 (2 μM) plus quinpirole (0.1 μM), as described in Fig. 1. Data are the mean$\pm$SE (bars) of 4 separate determinations from one transfection.

ability of D_2 receptors to facilitate evoked AA release, and raise the possibility that D_1-dependent activation of PKA may underlie the synergistic interaction between D_1 and D_2 receptors on AA release. In addition, the results suggest that stimulation of additional Gs-coupled receptors, colocalized with the D_2 subtype, might exert a similar synergistic action. For example, in brain amygdala and hypothalamus, high levels of both Gs-coupled PGE_2 receptors and Gi-coupled D_2 receptors have been shown to be expressed (Malet et al., 1982; Watanabe et al., 1985; Bouthenet et al., 1991).

4. Prostaglandin E_2 as a Potential Paracrine Messenger in the Brain Induced by Dopaminergic Activation

Prostaglandins are thought to exert important neuromodulatory actions in the brain, including regulation of sleep-wake cycles and modulation of transmitter-regulated adenylyl cyclase activity (Hayaishi, 1989; Partington et al., 1980; Schaad et al., 1987; Weidenfeld et al., 1992). In light both of our results, showing that PGE_2 enhances AA signaling at the D_2 receptor (see above), and of the known colocalization of PGE_2 and D_2 receptors in certain brain areas, we have begun to study the possible interactions between these receptor subtypes on the AA response. Using CHO(D_2) cells, we carried out experiments aimed at determining whether: (a) PGE_2 is produced following D_2-receptor activation, and (b) release of this prostaglandin in the extracellular medium may play a positive feedback role, enhancing D_2 potentiation of evoked AA release through stimulation of cAMP formation.

When incubation media from CHO(D_2) cells, prelabelled with [^{3}H]AA and stimulated with A23187 (4 μM) plus quinpirole (1 μM), were analysed by HPLC, a major radioactivity peak coeluting with authentic PGE_2 was observed (Di Marzo and Piomelli, 1992). The radioactivity associated with this component was greater in incubations of cells stimulated with A23187 plus quinpirole than in incubations of cells stimulated with Ca^{2+} ionophore alone (1040 ± 415 vs 360 ± 97 cpm/dish, $n = 5$). No radioactive material coeluting with PGE_2 was released from control, unstimulated cells or from cells stimulated with A23187 plus quinpirole in the presence the cycloxygenase inhibitor indomethacin (4 μM). The identity of this component as PGE_2 was confirmed by treating the HPLC fractions containing the radioactive material with methanolic KOH, which selectively converts PGE_2 into PGB_2. HPLC analysis of the reaction mixture yielded a major radiolabeled component coeluting with authentic PGB_2. Moreover, radioimmunoassay (RIA) carried out on HPLC fractions from incubation media of unlabeled, stimulated CHO(D_2) cells yielded a main immunoreactive peak coeluting with PGE_2 (Di Marzo and Piomelli, 1992). RIA was also used to measure the amounts of immunoreactive PGE_2 produced by cells stimulated with ionophore and quinpirole. The value obtained in stimulated cells (25.3 ± 7.1 vs 3.3 ± 1.1 nM in unstimulated cells, $n = 3$) was above threshold for PGE_2-induced potentiation of the

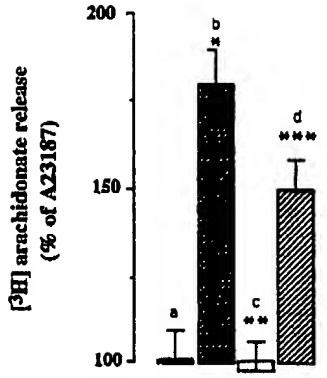

Fig. 7. Indomethacin prevents the potentiating effect of D_2 receptor stimulation on [^{3}H]AA release, and exogenous PGE_2 restores it. Prelabeled CHO(D_2) cells were incubated in 0.75 ml DMEM without BSA in the presence of A23187 (4 μM) plus indomethacin (4 μM) (column a), A23187 plus quinpirole (0.5 μM) (column b), A23187 plus quinpirole and indomethacin (4 μM) (column c), and the same plus PGE_2 (1 μM) (column d). Results are expressed as percentages of the release induced by A23187 and are mean ± SE (bars) values from 5 experiments carried out in quadruplicate. * $P < 0.05$ vs A23187 only; ** $P < 0.05$ vs A23187 plus quinpirole; *** $P < 0.05$ vs A23187 plus quinpirole and indomethacin. (Reproduced, with permission, from the Journal of Neurochemistry.)

quinpirole AA response. Thus, the results suggest that the levels of PGE_2 attained following activation of D_2-receptors may be sufficient to enhance AA release.

One prediction of this hypothesis is that blockade of PGE_2 formation should result in a decreased overall production of AA. This prediction was confirmed in experiments in which CHO(D_2) cells were stimulated with quinpirole plus ionophore in the presence of indomethacin (4 μM). The cycloxygenase inhibitor significantly inhibited quinpirole enhancement of AA liberation, and this inhibition was reversed by adding back PGE_2 (Fig. 7). The results support the idea that PGE_2, produced by D_2 receptor stimulation and diffused extracellularly, may act to modulate the D_2 response by binding to Gs-coupled receptors located on the same cell or on neighboring cells.

5. Conclusions

The experiments reported here leave open two major questions. First, the site of cAMP- and PKA-mediated control over AA signaling at the D_2 receptor remains to be determined. The results showing that neither increased cAMP levels nor PKA overexpression lead to any significant stimulation of PLA_2 in the absence of D_2-receptor occupation, suggest that the activity of PLA_2 is not directly controlled by PKA phosphorylation. Rather, PKA may act by increasing the productive coupling between D_2 receptors and the transducing G_i protein (Felder et al., 1991; Piomelli et al., 1991), possibly by catalysing phosphorylation of the ubiquitous G protein regulatory protein, phosducin (Bauer et al., 1992).

Another important question which remains to be addressed is whether our results, obtained in transfected CHO cells, are physiologically relevant to the CNS. Several lines of evidence suggest that this is likely be the case. In brain and in other tissues, stimulation of D_2 receptors may be linked to release and metabolism of AA. In hypothalamic median eminence, epoxygenase metabolites of AA have been implicated in D_2 release of somatostatin (Junier et al., 1990). In kidney renal medulla, a D_2-like receptor linked to the formation of PGE_2 has been described (Huo et al., 1990), and in rat brain synaptosomes dopamine (100 μM) was reported to evoke release of immunoreactive PGE_2 (Hillier et al., 1976). It will be important to determine the potential signaling role of the AA cascade in functional models of D_1/D_2 receptor synergism, such as the inhibition of firing in nucleus accumbens neurons (Clark and White, 1987) or the expression of striatal long-term depression (Calabresi et al., 1992a,b).

Acknowledgements

We wish to thank Dr R. Maurer for the generous gift of PKI plasmid and Dr G.S. McKnight for MTC-EVα and MTR-EV_{AB} plasmids. We thank also Drs P. Greengard and J.C. Schwartz for discussion, and D. Vial and Dr P. Sokoloff for help with some experiments. D.P. was supported by a Young Investigator Award from the National Alliance for Research on Schizophrenia and Depression and V.D.M. by a Short Term Fellowship from the European Molecular Biology Organization (EMBO).

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Volume 270, Number 25, Issue of June 23, pp. 14843-14846, 1995

How MAP Kinases Are Regulated (*)

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INTRODUCTION

The closely related MAP kinases,[1] (1)extracellular signal-regulated protein kinases 1 and 2 (ERK1 and ERK2), are ubiquitous components of signal transduction pathways. ERK1 and ERK2 are activated by diverse extracellular stimuli and by protooncogene products that induce proliferation or enhance differentiation (reviewed in Refs. 1 and 2). MAP kinase phosphorylations have an impact on processes in the cytoplasm, the nucleus, the cytoskeleton, and the membrane. The variety of signals that conscript the MAP kinase pathway demonstrates that this cascade serves a myriad of purposes, and the consequences of its activation will depend on cellular context. Because of the pleiotropic potential of these kinases, their activation needs to be tightly controlled. This review discusses the complexity of upstream regulation of the MAP kinase pathway, parallel cascades, and concepts that are likely to apply to many MAP kinase family members developed from analysis of the crystal structure of ERK2.

Control of the MAP Kinase Cascade

Receptor Tyrosine Kinases

The best understood means of activating the MAP kinase pathway (reviewed in Refs. 1 and 3) is that used by receptor tyrosine kinases. Ligands cause receptors to autophosphorylate on tyrosine residues; the phosphotyrosine residues of autophosphorylated receptors then bind the SH2 domains of adapters, such as Grb2 (growth factor receptor-bound protein 2). The adapters recruit guanine nucleotide exchange factors with proline-rich SH3 domain-binding sites to the membrane in proximity to the isoprenylated small G proteins they activate. Exchange factors promote the association of Ras with GTP. The GTP-bound form of Ras binds the protein kinases Raf-1 and B-Raf, thereby targeting one or both Raf isoforms to the membrane where Raf protein kinase activity is increased. MAP kinase kinases 1 and 2 (MKK), also called MAP/ERK kinases (MEK)(4, 5, 6) , are phosphorylated and activated by Raf-1 and B-Raf and are the upstream activators of the MAP kinases. Receptor tyrosine kinases have also been reported to activate the cascade in rat fibroblasts via a Ca^{2+}-dependent but protein kinase C (PKC)- and Ras-independent pathway(7) . Receptors that do not contain intrinsic tyrosine kinase activity but that harbor sites for tyrosine phosphorylation may also activate the cascade via association of phosphotyrosine residues on the receptors or the activated tyrosine kinases with adapters(8) .

G Protein-coupled Receptors

The MAP kinase cascade can also be activated by certain heterotrimeric G proteins(9, 10) . Most require Ras and are believed to exploit the steps described for tyrosine kinases, but Ras-independent activation has been reported (9-12).

PKC

PKC is used by many receptors types to regulate the MAP kinase pathway, alone or with other mechanisms(13, 14) , and may act at several steps in the cascade. The effects of phorbol esters are Ras-dependent in PC12 cells (11) and Jurkat cells (15) but Ras-independent in fibroblasts(16) , consistent with multiple sites of action of PKC. PKC may directly activate Raf-1(17) , but mutation of the site phosphorylated by PKC does not interfere with activation of Raf by many stimuli including phorbol ester(18) . Other sites of action of PKC are likely to be either farther upstream or at the level of MAP kinase inactivation.

Regulation and Specificity of MEKs

All known signaling pathways are believed to use the two dual specificity protein kinases MEK1 and MEK2 to phosphorylate and activate MAP kinase(6) . MEK1 and -2 are activated not only by Raf-1 and B-Raf (19, 20) but also by the Mos protooncogene product(21, 22) , MEK kinase 1 (MEKK1)[2][2](23) , and other probably distinct, growth factor-stimulated activities(24, 25) . The mechanisms controlling MEKK1 are unknown, although Ras may be required(26) . In oocytes, Mos is believed to be controlled by its synthesis and degradation.

Because no other MEK substrates have been identified, MEKs are viewed as dedicated kinases that phosphorylate only the MAP kinases. Kinases related to ERK1 and ERK2, in spite of retaining a similar arrangement of activating phosphorylation sites (Fig. 1), are poor *in vitro* substrates of MEK1(27) . Thus, the marked specificity of MEKs contributes to the selective activation of their downstream targets.

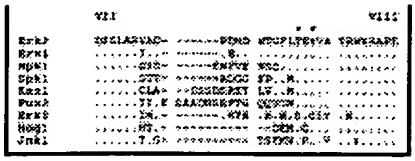

Figure 1: Alignment of phosphorylation lip sequences of ERK/MAP kinase family members. ERK1, ERK2, ERK3, HOG1, and JNK1 are mammalian enzymes. MPK1, KSS1, and FUS3 are from budding yeast and SPK1 is from fission yeast. The lip sequence of the *Drosophila rolled* gene product is identical to ERK1. *Dots* indicate identities; *dashes* indicate deletions. The phosphorylation sites are denoted by an *asterisk*. The 17 residues disordered in the ERK2 Tyr-185 mutants extend from the Asp preceding the FUS3 insertion to the conserved Arg preceding the sequence WYRAPE.

Parallel MAP Kinase Pathways

The ERK Protein Kinase Subfamily

ERK1 and ERK2 were the first of the ERK/MAP kinase subfamily to be cloned(28, 29, 30) . Other related mammalian enzymes have been detected including: two ERK3 isoforms(29, 31, 32) , ERK4(33) , Jun N-terminal kinases/stress-activated protein kinases (JNK/SAPKs)(34, 35) , p38/HOG1(36, 37) , and p57 MAP kinases (38). The presence of at least six MAP kinases in yeast suggests that there are more in mammals. Sequence signatures of the ERK family are most apparent in subdomains V, VII, IX, and XI (39) and include a long insert between subdomains X and XI. The sequences of the regulatory phosphorylation lip (surface loop between subdomains VII and VIII, see below) are also related, with conserved dual phosphorylation sites (Fig. 1).

The MEK Protein Kinase Subfamily

Several laboratories have uncovered additional MEKs, for which some substrates have been defined. A mammalian homolog of a MEK first identified in *Xenopus* (40) is called MAP kinase kinase 4 (MKK4), SAPK/ERK kinase (SEK), or JNK kinase (JNKK), because *in vitro* it activates JNK/SAPK and p38/HOG1 (27, 41, 42) but not ERK1 or ERK2(27) . Yet another newly cloned MEK, MKK3, selectively activates p38/HOG1 in transfected cells (42).

MAP Kinase Modules Mediate Distinct Signaling Events

The consistent appearance of 3-kinase cascades, first recognized in yeast, has engendered the concept of distinct *MAP kinase modules*(43) (Fig. 2). The modules convey information to target effectors and coordinate incoming information from parallel signaling pathways. A canonical MAP kinase module consists of three protein kinases that act sequentially within one pathway: a MEKK (a MEK activator), a MEK (a MAP kinase activator), and a MAP kinase (any ERK homolog). Raf-1 (or B-Raf), MEK1 (or MEK2), and ERK2 (or ERK1) constitute the best known mammalian MAP kinase module. The second mammalian MAP kinase module to be defined apparently consists of MEKK1, MKK4 (the MEK), and SAPK/JNK (the ERK)(44, 45) . MKK3 and p38/HOG1 appear to define yet a third cascade. MEKK1 can activate MKK4, MKK3, MEK1, and MEK2(27, 46, 47) , suggesting that MEKKs have a broader substrate specificity than MEKs. Thus, enzymatic specificity of the MEK, not the MEKK, may limit cross-cascade noise. Additional contributions to specificity may be provided through subcellular targeting of the enzymes(48) .

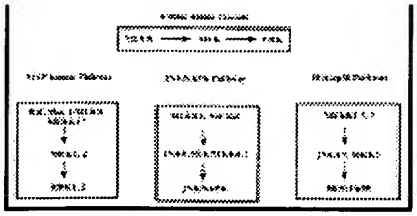

Figure 2: Mammalian MAP kinase modules. There are multiple MAP kinase modules in mammalian cells. Three that can be distinguished at present are the MAP kinase pathway, the JNK/SAPK pathway, and the HOG/p38 pathway. A MAP kinase module is a 3-kinase cascade consisting of an ERK or MAP kinase, which is activated by a MEK or MAP kinase kinase that in turn is activated by a MAP kinase kinase kinase or MEKK.

Activation and Inactivation of the MAP Kinases

Phosphorylation by MEK on two sites is required for MAP kinase activation. The two activating phosphorylation sites, a tyrosine and a threonine (Tyr-185 and Thr-183 of ERK2, Fig. 2and 3), lie 1 residue apart on the MAP kinases (49) in the phosphorylation lip. *In vivo* and *in vitro*, phosphorylation of tyrosine precedes phosphorylation of threonine(50, 51) , although phosphorylation of either residue can occur in the absence of the other(52, 53) . Because both tyrosine and threonine phosphorylations are required to activate the MAP kinases, phosphatases that remove phosphate from either site will inactivate them. Certain dual specificity phosphatases selectively inactivate MAP kinases by dephosphorylating both sites (reviewed in Ref. 54).

Three-dimensional Structure of ERK2

General Features

The three-dimensional structure of the unphosphorylated form of ERK2 provides a picture of its low activity state(55) . It consists of a smaller N-terminal domain and a larger C-terminal domain connected by a linker or crossover region (Fig. 3), similar to other protein kinases. ATP binds at a site deep in the catalytic cleft, formed at the interface between the two domains, whereas protein substrates bind on the surface.

THIS FIGURE IS CURRENTLY UNAVAILABLE

We apologize for any inconvenience.

Figure 3: The positions of gain-of-function mutations of MAP kinase mapped onto the three-dimensional structure of ERK2. *Red* denotes oxygen atoms; all other atoms (C, N, S, H) except as noted below are shown in *purple*. *Yellow* indicates Thr-183 and Tyr-185, the phosphorylated side chains; *darkblue* denotes basic residues likely to be involved in binding the phosphorylated side chains. *Brightgreen* and *turquoise* indicate dominant and recessive mutations, respectively. The residue numbers of ERK2 corresponding to the mutations are indicated. *A*, standard kinase view (profile); *B*, a second view rotated ~80° looking into the phosphorylation lip.

Conformational Changes

Phosphorylation probably activates ERK2 by causing both global and local conformational changes. The two domains of ERK2 are rotated ~17° farther apart than these domains in the structure of cAMP-dependent protein kinase (cAPK) (56). Therefore, a rotation of the N- and C-terminal domains must occur to cause closure of the active site and align the catalytic residues.

In cAPK, a phosphothreonine residue located in the phosphorylation lip interacts with basic residues, one of which is located in the N-terminal domain, to stabilize the closed domain structure. Similar interactions are likely to stabilize the closed state of ERK2. A domain rotation within ERK2 would bring homologous basic residues, including Arg-65 in the N-terminal domain (Fig. 3), into position to bind the phosphate group on Thr-183.

The phosphorylation lip, which contains the Thr-183 and Tyr-185 phosphorylation sites, blocks access of substrates to the active site. The side chain of Tyr-185 lies buried near the active site, and its main chain occupies the substrate binding site. A local conformational change occurs upon phosphorylation, displacing Tyr-185 and creating a lip structure compatible with high catalytic activity.

A Possible Binding Site for Phosphate on Tyr-185

Arg-189 and -192, residues not highly conserved among the protein kinases, create an anion binding site (Fig. 3) on the surface of ERK2 near the phosphorylation lip(57) . In the refined, low activity structure this site was filled with a sulfate ion acquired during crystallization. Interaction of the phosphate group of Tyr-185 with these residues may help to stabilize the conformation of the lip in the active structure.

MAP Kinase Mutants and Structural Implications

The Phosphorylation Lip Controls the Activity of the MAP Kinases

Biochemical and structural analyses of mutations of the activating phosphorylation sites suggest how phosphorylation increases ERK2 activity. The structure of the ERK2 mutant T183E and its basal activity are similar to wild type, but it is activated ~100-fold following a single phosphorylation on Tyr-185(53) , suggesting that glutamate in part mimics the negative charge of threonine phosphate. The crystal structures of three ERK2 mutants at Tyr-185 (57) suggest changes in local conformation upon ERK2 activation. In these mutants, 15 residues of the phosphorylation lip from Asp-173 to Ala-187 (Fig. 1) are disordered(57) . Because any change to Tyr-185 introduces disorder into the low activity structure, Tyr-185 likely has an essential role in creating the low activity conformation.

The findings of these structural studies have important implications for the regulation of ERK2 and related kinases. The disorder observed in the mutants indicates that the phosphorylation lip is not a stable structure and suggests that modest amounts of binding energy are sufficient to induce conformational changes in this region(57) . The phosphorylation lip must acquire a different conformation to be phosphorylated by MEK and, after phosphorylation, another conformation that is compatible with high catalytic activity. Tyr-185 is buried in the low activity conformation of ERK2, yet in the activation process it is phosphorylated first. The binding energy provided by interaction of ERK2 with MEK may be sufficient to dislodge Tyr-185 from its buried position allowing it access to the active site of MEK.

Locations of Mutations Identified in Genetic Selections for Activated MAP Kinases

Thus far, no mutations have been identified that greatly increase MAP kinase activity *in vitro*; however, gain-of-function mutations have been found in two MAP kinases, the product of the *Drosophila rolled* gene (58) and FUS3(59) , a component of the pheromone response pathway in budding yeast. The mutations and the corresponding residues in ERK2 are listed in and displayed on the ERK2 structure in Fig. 3. The mutations are characterized as dominant or recessive.

The three recessive FUS3 mutations are buried in the N-terminal domain (Fig. 3). These are the least likely to affect interactions with other molecules. In ERK2 these residues are in close proximity and are involved in packing the β-ribbon (residues 6-18) that replaces the A helix found in cAPK. This ribbon contributes to the positioning and rigidity of the core β-sheet of the N-terminal domain and may influence the open conformation of the two domains in the inactive enzyme. These mutations may increase flexibility of this part of the molecule.

The dominant mutations lie on the surface and could involve interactions with other molecules. Here we have analyzed other possible effects of these dominant mutations. One FUS3 mutation (His-230 of ERK2) results in a loss of charge on the substrate binding face near the putative phosphotyrosine binding site and most likely affects interactions with substrates or regulators. A second FUS3 mutant, Glu-58 of ERK2, lies in a part of the structure unique to ERKs that replaces the B helix of cAPK. This region, near the putative phosphothreonine binding site, may be important for interactions in the activated structure(57) . A Val to Leu mutation (V171L in ERK2) in FUS3 lies at the beginning of the phosphorylation lip. The mutation may release steric constraint associated with a β-branched residue, influencing refolding of the lip. The mutation identified in the *rolled* gene product (Asp-319 in ERK2) is just C-terminal to the conserved protein kinase core near the crossover region between the N- and C-terminal domains. Asp-319 forms a network of ionic interactions with residues conserved among MAP kinases to create a hinge bridging the N- and C-terminal domains. Thus, this mutation may affect the domain structure or orientation.

Conclusion

Thus far, no constitutively active MAP kinases are known, despite attempts at their genetic selection and site-directed mutagenesis. Such failure suggests that cells cannot tolerate the continuous activity of MAP kinase. Constitutively active mutants of MEK transform cells and generate tumors in nude mice(60) . However, effects of activated MEKs could be compensated for in a regulatable fashion by increasing phosphatase activity to inactivate MAP kinases. Perhaps the catastrophe that a cell might encounter if MAP kinases were constitutively active accounts for the diabolically complex mechanisms to activate these protein kinases and the multiplicity of mechanisms to inactivate them.

Dominant mutations	Recessive mutations	
ERK2	FUS3	ERK2FUS3
Glu-58	D48N	Phe-17Y7H
Val-171	I161L	Val-19I9K
His-230	D227N	Cys-38C28Y
Asp-319	*rolled*	
	D334N	

Table: Gain-of-function mutations in MAP kinases

The gain-of-function mutations in FUS3 (59) and the *rolled* gene product (58) are listed with the corresponding residue numbers in ERK2 and are grouped as dominant or recessive.

FOOTNOTES

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This minireview will be reprinted in the 1995 Minireview Compendium, which will be available in December, 1995. Work from the authors' laboratories was supported by grants from the Welch Foundation (I1128 to E. J. G. and I1243 to M. H. C.), the National Institutes of Health (DK34128 to M. H. C. and DK46993 to E. J. G.), the Texas Advanced Research Program, and the Tobacco Research Council.

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1

The abbreviations used are: MAP kinase, mitogen-activated protein kinase; ERK, extracellular signal-regulated protein kinase; MKK, MAP kinase/ERK kinase, MEK (the same as MAP kinase kinase; MEKK, MEK kinase; JNK/SAPK, Jun-N-terminal kinase/stress-activated protein kinase; PKC, protein kinase C; cAPK, cyclic AMP-dependent protein kinase.

2

Because multiple isoforms of MEKK are likely to exist, the first isoform cloned will be referred to throughout as MEKK1.

ACKNOWLEDGEMENTS

We would like to thank Elliott Ross, Jessie Hepler, Meg Phillips, and Megan Robinson (UT Southwestern) for critical reading of the manuscript and suggestions about figures, members of the Goldsmith and the Cobb laboratories for their efforts and insights, and Jo Hicks for preparation of the manuscript.

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Article title	Coupling gene expression to cAMP signalling: Role of CREB and CREM
Article identifier	1357272598004271
Authors	Sassone-Corsi_P
Journal title	International Journal of Biochemistry and Cell Biology
ISSN	1357-2725
Publisher	Pergamon
Year of publication	1998
Volume	30
Issue	1
Supplement	0
Page range	27-38
Number of pages	12
User name	Adonis
Cost centre	Development
PCC	$20.00
Date and time	Wednesday, May 03, 2000 5:06:53 AM

PERGAMON

The International Journal of Biochemistry & Cell Biology 30 (1998) 27–38

Biochemistry & Cell Biology

Review

Coupling gene expression to cAMP signalling: role of CREB and CREM

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Abstract

Several endocrine and neuronal functions are governed by the cAMP-dependent pathway. Transcriptional regulation upon stimulation of this pathway is mediated by a family of cAMP-responsive nuclear factors. This family consists of a large number of members, which may act as activators or repressors. These factors contain the basic domain/leucine zipper motifs and bind as dimers to cAMP-response elements (CRE). CRE-binding protein (CREBs) function is modulated by phosphorylation by several kinases. Direct activation of gene expression by CREB requires phosphorylation by the cAMP-dependent PKA to serine 133. Among the repressors, ICER (Inducible cAMP Early Repressor) deserves special mention. ICER is generated from an alternative CREM promoter and is the only inducible CRE-binding protein. ICER negatively autoregulates the alternative promoter, generating a feedback loop. ICER expression is tissue specific and developmentally regulated. The kinetics of ICER expression are characteristic of an early response gene.

CREM plays a key physiological and developmental role within the hypothalamic–pituitary–gonadal axis. The transcriptional activator CREM is highly expressed in postmeiotic cells. The role of CREM in spermiogenesis was addressed using CREM knock-out mice. Spermatogenesis stops at the first step of spermiogenesis in the mutants and there is a significant increase in apoptotic germ cells. This phenotype is reminiscent of cases of human infertility.

ICER is regulated in a circadian manner in the pineal gland, the site of the hormone melatonin production. This night–day oscillation is driven by the endogenous clock (located in the suprachiasmatic nucleus). The synthesis of melatonin is regulated by a rate-limiting enzyme, serotonin *N*-acetyltransferase (NAT). Analysis of the CREM-null mice and of the promoter of the NAT gene revealed that ICER controls the amplitude and rhythmicity of NAT, and thus the oscillation in the hormonal synthesis of melatonin.

Keywords: Cyclic AMP; CREB; CREM; Phosphorylation; Autoregulation

1. Introduction

The regulation of gene expression by specific signal transduction pathways is tightly connected to the cell phenotype and, conversely, the response elicited by a given transduction pathway varies depending on the cell type. Several molecules implicated in intracellular signalling are encoded by oncogenes, directly linking their possible aberrant expression to cellular transformation or

1357-2725/98/$19.00
PII: S1357-2725(97)00093-9

altered proliferation. A complete analysis of these processes will help to unravel the profound changes that cause cancer and, by the same token, understand the physiology of normal growth. A fundamental stride has been the discovery that many transcription factors constitute final targets of specific transduction pathways. Many distinct kinases have been shown to directly or indirectly modulate the activity of various nuclear factors (Karin and Hunter, 1995).

The activity of transcription factor AP-1 may be increased by inducing c-*fos* gene transcription, a process mediated by the ERK-1 and -2 mitogen-activated protein (MAP) kinases, which directly phosphorylate the transcription factor Elk-1/TCF, which then binds to the c-*fos* serum response element (Treisman, 1996). Alternatively, AP-1 activity maybe be enhanced by direct phosphorylation of Jun by a different type of MAPKs, the stress-activated protein kinases (JNK/SAPK) (Davis, 1994). Transcription factor ATF-2, a dimerization partner of Jun, is also a target of the JNK kinase (Hazzalin et al., 1996). Interestingly, ATF-2 was first cloned as a member of the ATF/CREB family of transcription factors and was shown to bind to cAMP-responsive elements (CREs) (Hai et al., 1989). The ATF/CREB family includes several members, of which only the CREB, CREM and ATF-1 gene products have been shown to be directly phosphoryalted by the cAMP-dependent protein kinase A (Sassone-Corsi, 1995). Cross-talk between the mitogenic signalling pathways and cAMP-responsive transcription has been established (Ginty et al., 1994), which reinforces the notion of converging signalling within the PKA and PKC pathways in the cytoplasm (Cambier et al., 1987; Yoshimasa et al., 1987; Frodin et al., 1994) and in the nucleus (Masquilier and Sassone-Corsi, 1992).

An important example of signalling cross-talk in the nucleus involves the pathway coupled to the NGF receptor, Trk, which results in the activation of several kinases. Trk is a receptor tyrosine kinase which, once activated, stimulates the activity of the small GTP-binding protein Ras (Gomez and Cohen, 1991). Activation of Ras triggers the MAPK pathway, which includes the MAP kinase kinase (MEK) and the ribosomal S6 kinase $pp90^{rsk}$ (Cobb and Goldsmith, 1995). Interestingly, constitutively activated expression of MAPK and MEK is sufficient to induce neurite outgrowth in PC12 cells (Cowley et al., 1994; Fukuda et al., 1995), indicating a direct role of this pathway in eliciting the changes in gene expression required for the neuronal differentiation program. Although MAPK and MEK have not been shown to directly phosphorylate CREB, the use of cells expressing a dominant-interfering Ras mutant has revealed the involvement of this pathway for CREB phosphorylation upon NGF-induction (Ginty et al., 1994). Indeed, the involvement of a CREB-kinase which could have characteristics similar to $pp90^{rsk}$ has been proposed (Fig. 1). $pp90^{rsk}$ is likely to be responsible for CREB phosphorylation in human melanocytes (Böhm et al., 1995), while the other member of the RSK family, $p70^{s6k}$, also possesses CREB phosphorylation activity (de Groot et al., 1994). Thus, two different signalling pathways may converge to modulate gene expression via the same transcriptional regulator, CREB (Fig. 1). Finally, CREB has been shown to be phosphorylated upon activation of the stress pathway involving the p38/MAPKAP2 kinases (Tan et al., 1996).

The complexity of the signalling pathways controlling transcription factors is a demonstration of the pleiotropic functions played by these molecules in the regulation of physiology and metabolism. Here we will focus primarily on the targets of the cAMP-mediated transduction response and their function within the neuroendocrine response.

2. Phosphorylation: a prerequisite for activation

Intracellular levels of cAMP are regulated primarily by adenylyl cyclase. This enzyme is, in turn, modulated by various extracellular stimuli mediated by receptors and their interaction with G proteins (McKnight et al., 1988). The binding of a specific ligand to a receptor results in the activation or inhibition of the cAMP-dependent pathway, ultimately affecting the transcriptional

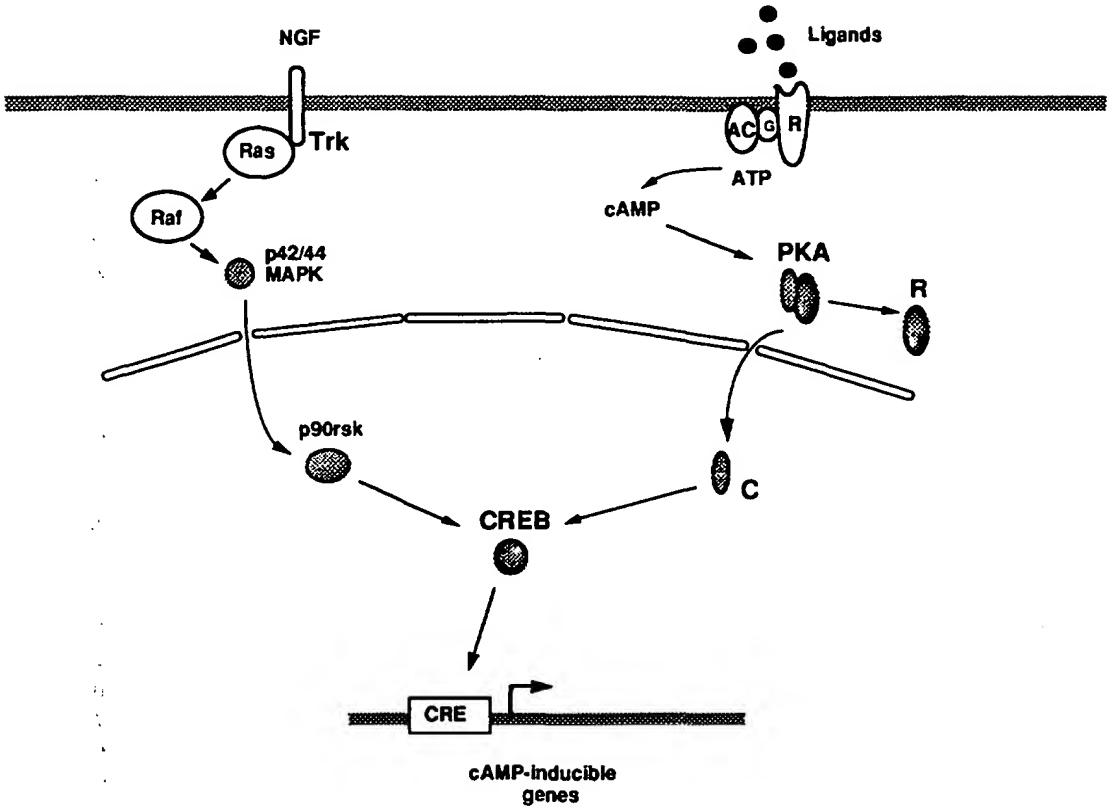

Fig. 1. Cross-talk in signal transduction. Schematic representation of the route whereby ligands at the cell surface interact with membrane receptors (R) and thereby result in altered gene expression upon activation of the cAMP signal transduction pathway. Ligand binding activates coupled G-proteins (G) which, in turn, stimulate the activity of the membrane-associated adenylyl cyclase (AC). This converts ATP to cAMP, which causes the dissociation of the inactive tetrameric protein kinase A (PKA) complex into the active catalytic subunits and the regulatory subunits. Catalytic subunits (C) migrate into the nucleus, where they phosphorylate and thereby activate transcriptional activators such as CREB. CREB then interacts as a dimer with the cAMP response enhancer element (CRE) found in the promoters of several cAMP-responsive genes to activate transcription. CREB phosphorylation may be obtained also by activation of the NGF (Nerve Growth Factor) tyrosine kinase receptor Trk. This pathway involves the Ras–Raf signalling cascade and results in the activation of the RSK class of kinases. CREB can be phosphorylated at the same PKA-phosphoacceptor site (Ser-133) by $p90^{rsk}$. This phosphorylation event may result in the activation of cAMP-responsive gene expression via a cAMP-independent signalling cascade.

regulation of various genes through distinct promoter-responsive sites. Increased cAMP levels directly affect the function of the tetrameric protein kinase A (PKA) complex. Binding of cAMP to two PKA regulatory subunits releases the catalytic subunits, enabling them to phosphorylate target proteins. These are translocated from cytoplasmic and Golgi complex anchoring sites and phosphorylate a number of cytoplasmic and nuclear proteins on serines in the context X-Arg-*Arg-X-Ser*-X (McKnight et al., 1988; Roesler et al., 1988). A number of isoforms for both the regulatory and catalytic subunits have been identified, suggesting a further level of complexity in this response (McKnight et al., 1988). In the nucleus, the phosphorylation state of transcription factors and related proteins appears to directly modulate their function and thus the expression of cAMP-inducible genes. Thus, there is a direct link between the activation of G-coupled membrane receptors and CRE-mediated gene expression.

The analysis of regulatory sequences of several genes allowed the identification of promoter elements which mediate the transcriptional response to increased levels of intracellular cAMP (Lalli and Sassone-Corsi, 1994). A number of sequences have been identified, of which the best characterised is the CRE. A consensus CRE site constitutes an 8 bp palindromic sequence (TGACGTCA) (Sassone-Corsi, 1988; Ziff, 1990). Several genes which are regulated by a variety of endocrinological stimuli contain similar sequences in their promoter regions, although at different positions. A comparison of the CRE sequences identified to date, shows that the 5'-half of the palindrome, TGACG is the best conserved, differently from the 3' TCA motif (Sassone-Corsi, 1995).

The first CRE-binding factor to be characterised was CREB (CRE-binding protein; Hoeffler et al., 1988) but subsequently several additional CRE-binding factors have been identified and the corresponding gene cloned. Most of the CRE-binding proteins were identified by screening a variety of cDNA expression libraries with CRE and ATF sites (Hai et al., 1989; Foulkes et al., 1991). All these proteins belong to the bZip transcription factor class, while outside of the bZip region, sequence homology between these factors is relatively poor. Various different factors of the CREB/ATF family are able to heterodimerize with each other but only in certain combinations. A "dimerization code" exists, which seems to be a property of the leucine zipper structure of each factor.

CRE-binding proteins may act as both activators and repressors of transcription. The activators mediate transcriptional induction upon their phosphorylation by PKA (Gonzalez and Montminy, 1989; Rehfuss et al., 1991; de Groot et al., 1993; Sassone-Corsi, 1995). Their expression is constitutive and widely distributed in various tissues in a housekeeping fashion. Among the repressors, the cAMP-inducible ICER (Inducible cAMP Early Repressor) product deserves special mention. It is generated from a cAMP-inducible alternative promoter of the CREM gene (Molina et al., 1993; Stehle et al., 1993). Thus, ICER is an early response CRE-binding factor and is involved in the dynamics of cAMP-responsive transcription (Lamas et al., 1996).

3. Interaction with CBP

Further steps towards an understanding of the mechanism of action of the P-box have arisen with the identification of a 265 K, 2441 amino acid protein, CBP (CREB-binding protein) that is able to interact specifically with the phosphorylated CREB P-box domain (Chrivia et al., 1993). The CBP sequence reveals two zinc finger domains, a glutamine-rich domain at its C-terminus and a single consensus PKA recognition site. Phosphorylation of Ser-133 promotes binding to CBP and consequently the interaction with TFIIB, a general transcription factor involved in RNA polymerase II activity (Kwok et al., 1994). Thus, CBP may act as a link between CREB and the transcription pre-initiation complex. This interaction may reqire some RNA polymerase II cofactors, such as TAF110. Finally, the adenoviral E1A oncoprotein-associated p300, which is thought to play a role in preventing the cell cycle G0/G1 transition, is structurally very closely related to CBP (Arany et al., 1995). Both CBP and p300 appear to have intrinsic activating properties which are inhibited by the E1A protein (Arany et al., 1995). Thus, it is clear that studies of the transcriptional activation domain of CRE-binding bZip factors continue to provide important insights into the function of transcription factors in general.

4. Mechanisms of repression

Dephosphorylation appears to represent a key mechanism in the negative regulation of CREB activation function. It has been proposed that a mechanism to explain the attenuation of CREB activity following induction by forskolin is dephosphorylation by specific phosphatases (Hagiwara et al., 1992). After the initial burst of phosphorylation in response to cAMP, CREB is dephosphorylated *in vivo* by protein phosphatase-

1 (PP-1). However, the situation is more complex, since it has been shown that both PP-1 and PP-2A can dephosphorylate CREB *in vitro* (Nichols et al., 1992) resulting in an apparent decreased binding to low-affinity CRE sites *in vitro*. Therefore, the precise role of PP-1 and PP-2A in the dephosphorylation of CREB remains to be determined.

The discovery of the CREM gene opened a new dimension in the study of the transcriptional response to cAMP (Foulkes and Sassone-Corsi, 1992). The dynamic and versatile pattern of CREM expression combined with its tissue- and developmental-specific pattern, contrasts with that of the remaining members of the CRE-binding factor family, which seem to be constant and ubiquitous (Hai et al., 1989; Borrelli et al., 1992). These features offered the first clue that CREM occupied a priviliged position amongst this group of factors.

Various studies have established that differential transcript processing is central to the regulation of CREM expression. The importance of this mechanism is reinforced by the fact that all the CREM isoforms which incorporate the P-box exons (Fig. 2) are generated from a GC-rich promoter (P1), which has been shown to behave as a housekeeping promoter directing a non-inducible pattern of expression (Molina et al., 1993; Stehle et al., 1993).

5. An inducible repressor: ICER

An alternative promoter lying within an intron near the 3′ end of the CREM gene, directs the transcription of a truncated product, termed ICER (Inducible cAMP Early Repressor) (Molina et al., 1993; Stehle et al., 1993). The ICER open reading frame is constituted by the C-terminal segment of CREM (Fig. 2). The predicted open reading frame encodes a small protein of 120 amino acids with an expected molecular weight of 13.4 kDa. This protein, compared with the previously described CREM isoforms, essentially consists of only the DNA-binding domain, which consists of the leucine zipper and basic region. The unique structure of ICER is suggestive of its function and makes it one of the smallest transcription factors ever described (Molina et al., 1993; Stehle et al., 1993).

The intact DNA-binding domain directs specific ICER binding to a consensus CRE el-

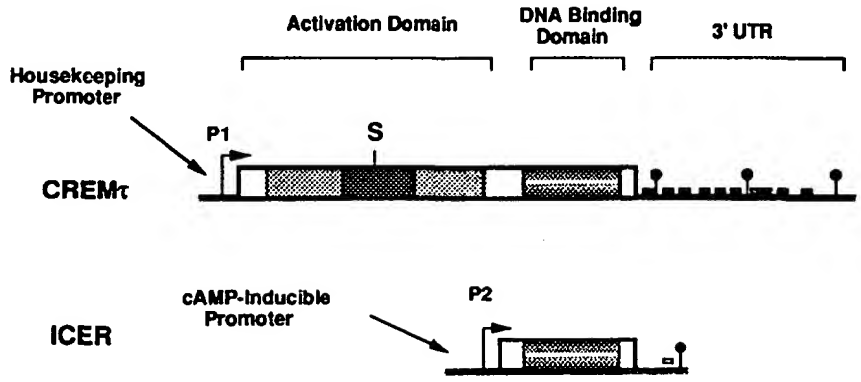

Fig. 2. Activators and repressors from the same gene. Schematic representation of the CREM gene. The various activator and repressor CREM isoforms are indicated. The P1 promoter is GC-rich and directs a non-inducible pattern of expression of the activator, CREMτ, which has a structure similar to CREB. The P2 promoter is strongly inducible by activation of the cAMP-dependent signalling pathway and directs the synthesis of the powerful repressor ICER. In germ cells the abundant CREMτ transcript is polyadenylated at an alternative site which confers increased stability. Schematic representation of the 3′ untranslated region (3′ UTR). The three polyadenylation signals (polyA) are non-canonical and are indicated by a stem-loop; each AUUUA destabiliser is represented by a small square. Use of the testis-specific site generates a transcript with a truncated 3′ untranslated region and only one instability element. This transcript is intrinsically more stable.

ement. Importantly, ICER is able to heterodimerize with the other CREM proteins and with CREB. ICER functions as a powerful repressor of cAMP-induced transcription in transfection assays using an extensive range of reporter plasmids carrying individual CRE elements or cAMP-inducible promoter fragments (Molina et al., 1993). Interestingly, ICER-mediated repression is obtained at substoichiometric concentrations, similarly to the previously described CREM antagonists (Laoide et al., 1993). ICER escapes from PKA-dependent phosphorylation and thus constitutes a new category of CRE binding factor, for which the principle determinant of their activity is their intracellular concentration and not their degree of phosphorylation.

The expression of ICER was first described in the pineal gland, where it is the subject of a dramatic circadian pattern of expression (Stehle et al., 1993). Additional data implicate dynamic ICER expression as a general feature of neuroendocrine systems (Lamas and Sassone-Corsi, 1996). An important feature about ICER is its inducibility. This makes ICER the only CRE-binding protein whose function is physiologically regulated by altering its cellular concentration.

6. CREM is an early response gene

During studies of CREM expression within the neuroendocrine system, an unexpected new facet emerged: namely the transcription of the CREM gene is inducible by cAMP (Molina et al., 1993). Furthermore, the kinetics of this induction is that of an early response gene (Verma and Sassone-Corsi, 1987). This important finding further reinforces the notion that CREM products play a fulcral role in the nuclear response to cAMP, since the expression of no other CRE-binding factor has been shown to be inducible to date. For example, the recently characterised CREB promoter is GC-rich and reminiscent of the promoters of constitutively expressed, housekeeping genes (Meyer et al., 1993). Similarly, the promoter which directs expression of the other CREM isoforms (P1) is not cAMP inducible (Molina et al., 1993).

Clues that the CREM gene was cAMP inducible first came from the demonstration that adrenergic signals direct CREM transcription in the pineal gland (Stehle et al., 1993). The inducibility phenomenon was then characterised in detail in the pituitary corticotroph cell line AtT20. In unstimulated cells the level of CREM transcript is below the threshold of detectability. However, upon treatment with forskolin (or other cAMP analogs), within 30 min there is a rapid increase in CREM transcript levels, which peak after 2 h and then progressively decline to basal levels by 5 h. This characteristic expression profile classifies CREM as an "early response gene" and thus directly implicates the cAMP pathway in the cell's early response for the first time. CREM inducibility is specific for the cAMP pathway, since it is not inducible by TPA or dexamethasone treatment (Molina et al., 1993).

The 5′ end of the ICER clones correspond to an alternative transcription start site. The start of transcription, which identifies the so-called P2 promoter, is within the 10 kb intron which is C-terminal to the Q2 glutamine-rich domain exon. In contrast to the promoter which generates all the previously characterised CREM isoforms (P1) which is GC-rich and not inducible by cAMP (unpublished results), the P2 promoter has a normal A-T and G-C content and is strongly inducible by cAMP. It contains two pairs of closely spaced CRE elements organized in tandem, where the separation between each pair is only three nucleotides. These features make P2 unique amongst cAMP-regulated promoters and are suggestive of cooperative interactions among the factors binding to these sites.

7. A negative autoregulatory loop

Upon cotreatment with cycloheximide, the kinetics of CREM gene induction by forskolin are altered in that there is a significant delay in the post-induction decrease in the transcript; elevated levels persist for as long as 12 h. This implicates a *de novo* synthesised factor which might downregulate CREM transcription (Molina et al., 1993). This observation, combined with the presence of

CRE elements in the P2 promoter, suggested that the transient nature of the inducibility could be due to ICER. Consistently, the CRE elements in the P2 promoter have been shown to bind to the ICER proteins. Detailed studies have demonstrated that the ICER promoter is indeed a target for ICER negative regulation (Molina et al., 1993; Lamas et al., 1996). Thus, there exists a negative autoregulatory mechanism controlling ICER expression. The CREM feedback loop predicts the presence of a refractory inducibility period in the gene's transcription (see Fig. 3; Sassone-Corsi, 1994).

8. Role of CREM in spermatogenesis

CREM is a highly abundant transcript in adult testis, while in prepubertal animals it is expressed at very low levels. Thus, CREM is the subject of a developmental switch in expression in testis (Foulkes et al., 1992). Further charaterisation revealed that the abundant CREM transcript encodes the activator exclusively, while in prepubertal testis only the repressor forms were detected at low levels. Thus, the CREM developmental switch also constitutes a reversal of function (Foulkes and Sassone-Corsi, 1992).

Spermatogenesis is a process occurring in a precise and coordinated manner within the seminiferous tubules (Jégou, 1993). During this entire developmental process the germ cells are maintained in intimate contact with the somatic Sertoli cells. As the spermatogonia mature, they move from the periphery towards the lumen of the tubule until the mature spermatozoa are conducted from the lumen to the collecting ducts.

CREM activator protein is detected in mature germ cells, such as round spermatids, which have undergone meiosis (Delmas et al., 1993). Thus, CREM transactivator function must be restricted to the late phase of transcription before the com-

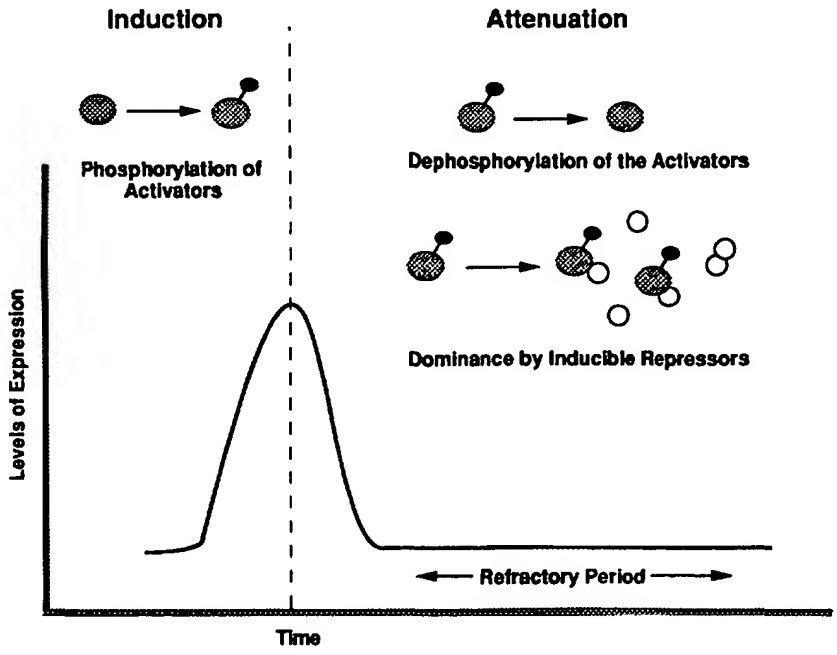

Fig. 3. Kinetics of CREM inducibility. After the induction phase, owing to the phosphorylation of the activators (i.e. CREB), expression is attenuated by at least two mechanisms: (a) dephosphorylation of the activators by some specific phosphatases; (b) negative autoregulation by the *de novo* synthesised ICER repressor on the P2 promoter (see Fig. 2) (Molina et al., 1993; Sassone-Corsi, 1994).

paction of the DNA. Interestingly, several genes have been identified which are transcribed at the time of appearance of the CREM protein and which include CRE-like sequences in their promoter regions. Several lines of evidence demonstrate that CREM constitutes the first step of a transcriptional cascade which is responsible for the activation of several germ-specific genes. To date, at least three genes, RT7 (Delmas et al., 1993), transition protein-1 (Kistler et al., 1994) and claspermin (Sun et al., 1995) have been shown to be targets of CREM-mediated transactivation in germ cells. Importantly, the dramatic increase in the levels of CREM protein correlates with its concomitant phosphorylation at serine 117 by a cAMP-stimulated PKA activity in round spermatid extracts (Delmas et al., 1993). Thus, CREM appears to participate in the testis-specific promoter activation of numerous haploid-expressed genes (Sassone-Corsi, 1997).

A remarkable aspect of the CREM developmental switch in germ cells is constituted by its exquisite hormonal regulation. The spermatogenic differentiation program is under the tight control of the hypothalamic pituitary axis (Jégou, 1993). The regulation of CREM function in testis seems to be intricately linked to FSH, both at the level of the control of transcript processing and at the level of protein activity. For example, surgical removal of the pituitary gland leads to the loss of CREM expression in the rat adult testis (Foulkes et al., 1993). Furthermore hypophysectomisation in prepubertal animals, prevents the switch in CREM expression at the pachytene spermatocyte stage, thus implicating the pituitary directly in the maintenance of, as well as the switch to high levels of CREM expression. Injections of FSH lead to a rapid and significant induction of the CREM transcript. The hormonal induction of CREM by FSH is not transcriptional, as expected by the housekeeping nature of the P1 promoter. Instead, by a mechanism of alternative polyadenylation, AUUUA destabiliser elements present in the 3′ untranslated region of the gene are excluded, dramatically increasing the stability of the CREM message (Fig. 2). CREM is the first example of a gene whose expression is modulated by a pituitary hormone during spermatogenesis (Foulkes et al., 1993). The implication of these findings is that hormones can regulate gene expression at the level of RNA processing and stability.

To address the role of CREM in development and in physiological processes we generated mutant mice with a gene disrupted by homologous recombination in mouse embryonic stem cells (Nantel et al., 1996). We constructed a targeting vector containing a CREM genomic fragment in which a portion of the 3′-terminal exon encoding the DNA-binding domain was deleted and replaced by a PGK-neomycin cassette. The selection of the construct was dictated by the need to inactivate all the numerous CREM and ICER isoforms (Laoide et al., 1993; Stehle et al., 1993). Reduced fertility was observed in the breeding of the heterozygous mice. Comparison of the homozygous CREM-deficient mice with their normal littermates showed no macroscopic physical aberrations or reduction in body weight. Analysis of internal organs revealed no apparent changes in their structure as compared with wild-type mice. However, the testes of the CREM-deficient mice displayed a reduction of 20–25% in their weight. Analysis of the seminal fluid of heterozygous mice compared with normal littermates demonstrated a 46% reduction in the overall number of spermatozoa, a 35% decrease in the ratio of motile spermatozoa, and a 2-fold increase in the number of spermatozoa with aberrant structures. Most of the aberrant spermatozoa were characterized by a kink and bubble-like structure midway along the tail. Strikingly, analysis of the seminal fluid from homozygous CREM-deficient mice revealed a complete absence of spermatozoa. This result demonstrates a dramatic impairement of spermatogenesis in the CREM-deficient mice. The homozygous female mice were fertile and displayed apparently normal ovary structure.

To determine the nature of the sperm deficiency in the CREM-deficient mice, we performed a detailed anatomical analysis of the seminiferous epithelium. Consecutive spermatogenic cycles are classically depicted as waves of differentiating germ cells within each tubule

(Parvinen, 1993). In the mouse, each wave is divided into 12 stages, each representing a specific cellular association. Tubular segments containing postmeiotic germ cells which undergo spermiogenesis appear as dark sections under transillumination because of the higher DNA compaction of these haploid cells (Parvinen, 1993). Tubuli from CREM-deficient mice display a 20–30% reduced diameter and completely lack the normal spermatogenic wave and the corresponding dark sections. Squash preparations from consecutive segments of the seminiferous epithelium demonstrate that spermatogenesis in the CREM-deficient mice is interrupted at the stage of very early spermatids. Neither elongating spermatids, nor spermatozoa, are observed, while somatic Sertoli cells appear to be normal.

9. Role of CREM in circadian rhythms

Crucial elements for the synchronization of biological rhythms in mammals are the pineal gland (Tamarkin et al., 1985) and the suprachiasmatic nucleus (SCN) (Moore, 1983). Environmental lighting conditions are transduced by the pineal gland from a neuronal to an endocrine message, the rhythmic secretion of melatonin (Tamarkin et al., 1985). This hormone synthesis is controlled by the SCN, being elevated at night and low during the day (Moore, 1983). The cAMP-dependent signal transduction pathway serves as a relay to stimulate melatonin synthesis. Thus, from neuronal pathways which include the retina and the SCN, the pineal gland acts as a temporal regulator of the hypothalamic–pituitary–gonadal axis (Tamarkin et al., 1985).

The study of CREM expression in the rat brain indicated a specific pattern of expression (Mellström et al., 1993). Analysis of CREM expression in the pineal gland has revealed a dramatic day–night regulation, with peak during the night. The CREM isoform in the pineal gland corresponds to ICER, the early response repressor known to be cAMP-inducible in endocrine cells (Stehle et al., 1993). The transcript shows a very characteristic and reproducible kinetic of expression. It appears likely that the autoregulatory loop shown to control ICER transient inducibility would also play a role in the day–night cyclic expression in the pineal gland.

The mechanism controlling this pattern of ICER expression was determined and found to require clock-distal elements. Indeed, it is known that at night, postganglionic fibers originating from the superior cervical ganglia (SCG) release norepinephrine, which in turn regulates melatonin synthesis via adrenergic receptors. These analyses have shown that signals from the SCN direct the induction of CREM expression (Stehle et al., 1993).

The question of possible targets for downregulation by ICER in the gland is of particular interest. It has been proposed that a reasonable target could be the enzyme which catalyses the rate-limiting step of melatonin synthesis, namely *N*-acetyl transferase (NAT) or factors which regulate its activity. Recent results indicate that this is indeed the case. The NAT promoter was shown to contain a CRE which binds ICER with high affinity. In addition, the amplitude of NAT oscillation in CREM-deficient mice was shown to be altered with respect to wild-type animals, demonstrating that NAT is a direct target of CREM (Foulkes et al., 1996).

Another important finding concerning the role of CRE-binding factors in circadian rhythms concerns the cyclic phosphorylation of CREB in the suprachiasmatic nucleus (Ginty et al., 1993). During the night, upon light stimuli which phase-shift the clock, CREB appears to be efficiently phosphorylated by an SCN-endogenous kinase at the serine 133 residue. Phosphorylation at this site turns CREB into an activator and may be obtained by a number of kinases (Lalli and Sassone-Corsi, 1994). While the nature of the SCN-endogenous kinase has not been established, it seems likely that it could be PKA (Ginty et al., 1993). This result would suggest a key role for this kinase or of a counteracting phosphatase in the regulation of the clock function. The target genes for the activated CREB in the SCN have yet to be established.

10. Conclusions and perspectives

The cAMP signal transduction pathway plays a key role in many biological processes. In the mammalian neuroendocrine system, it is central to the coordination of hormonal function. cAMP directs changes in gene expression and thereby effects long-term modulation. A great diversity of cAMP responsive transcription factors, notably CREM, seems to be a hallmark of this system. Many issues still remain to be explored in delineating the means by which CRE-binding proteins regulate complex phenomena such as memory formation and establishment. However, the abundance of molecular tools now available should aid this task. By understanding the precise mode of action of cAMP we should gain a more general insight into the molecular architecture which underlies physiology.

Acknowledgements

I would like to thank all the members of my research team for discussions and support. Work in my laboratory is funded by grants from Centre National de la Recherche Scientifique, Institut National de la Santé et de la Recherche Médicale, Centre Hospitalier Universitaire Régional, Rhône-Poulenc Rorer (Bioavenir), Fondation de la Recherche Médicale and Association pour Recherche sur le Cancer.

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Himmler et al J. Receptor Research 13(1-4)79-94, 19994

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Article title	Functional Coupling of Human Adenosine Receptors to a Ligand-Dependent Reporter Gene System
Article identifier	0006291X9400224M
Authors	Castanon_M_J Spevak_W
Journal title	Biochemical and Biophysical Research Communications
ISSN	0006-291X
Publisher	Academic Press USA
Year of publication	1994
Volume	198
Issue	2
Supplement	0
Page range	626-631
Number of pages	6
User name	Adonis
Cost centre	Development
PCC	$30.00
Date and time	Wednesday, May 03, 2000 2:58:22 PM

Vol. 198, No. 2, 1994
January 28, 1994

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 626-631

FUNCTIONAL COUPLING OF HUMAN ADENOSINE RECEPTORS TO A LIGAND-DEPENDENT REPORTER GENE SYSTEM

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Received December 13, 1993

We have stably transfected CHO cells that have integrated in their genome a reporter gene under the control of promoter sequences containing several copies of the cAMP response element, CRE, with different human adenosine receptors: A1, A2a and A2b. The new cell lines responded to the addition of known adenosine agonists and antagonists with changes in the expression of the reporter gene. The activity of the reporter gene can be easily monitored by bioluminescence. Although adenosine receptors are divergently coupled to adenylate cyclase, A1 receptors inhibit whereas A2 stimulate, changes in gene expression faithfully reflected the negative and positive coupling of the receptors. We have used the system to examine the pharmacological profile of the human receptors expressed in CHO cells.

The regulatory actions of adenosine, an important cellular messenger that modulates neuronal activity, smooth muscle tone, renal renin release, platelet aggregation, and lymphocyte function, are mediated by the adenosine receptors (1-3). These receptors belong to the family of G protein coupled receptors and utilize adenylate cyclase as the effector system. On the basis of their opposite action on adenylate cyclase the receptors were originally classified into two major classes, A1 and A2. Activated A1 receptors inhibit adenylate cyclase activity whereas A2 receptors stimulate the activity of the enzyme. A2 receptors are further subdivided into high affinity, A2a, and low affinity, A2b, receptor subtypes. A1 and A2 adenosine receptors also differ in primary sequence, tissue distribution, and binding affinities for synthetic adenosine analogues (3-5).

Molecular biology has brought radical changes in the study of receptors. Cloned receptors have enabled a clear cut identification of distinct receptor subtypes and their expression in transfected cell lines provided a powerful tool for studying receptor functions, highlighting pharmacological differences between receptor subtypes, and designing new and more selective ligands (6). Cells

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Abbreviations: ADSPX, 1-allyl-3,7-dimethyl-8-*p*-sulfophenyl-xanthine; AM, theophylline ethylenediamine; CPA, N^6-cyclopentyladenosine; CHA, N^6-cyclohexyladenosine; CCPA, 2-chloro-N^6-cyclopentyladenosine; CPCA, 5'-(N-cyclopropyl)-carboxamidoadenosine; CPT, 8-cyclopentyl-1,3-dimethylxantine; DMPX, 3,7-dimethyl-1-(2-propynyl) xantine; DPMA, N^6-[2-(3,5-dimetoxyphenyl)-2-(2-methylphenyl)-ethyl]adenosine; DPMX, 1,3-dipropyl-7-methyl-xanthine; DPX, 1,3-diethyl-8-phenylxanthine; NECA, 5'-N-ethylcarboxamidoadenosine; PACPX, 1,3-dipropyl-8-(2-amino-4-chlorophenyl)xanthine; R-PIA, N^6-(2-phenylisopropyl)-adenosine; 8-PT, 1,3-dimethyl-8-phenylxanthine XAC, xanthine amino congener.

0006-291X/94 $5.00

expressing cloned receptors can be used in functional screenings based on a broad view of receptor function, rather than on competitive binding. Prototypical functional assays for receptors are based on monitoring activation of a signal transduction pathway at either end of the cascade, such as the accumulation of a second messenger or changes in gene expression (7). We report here the heterologous expression of A1 and A2 adenosine receptors in CHO cells and their functional coupling, negative and positive, respectively, to adenylate cyclase as measured by a transcription assay. This assay is based on the induction of a reporter gene, luciferase, which has been fused to promoter sequences containing the regulatory cAMP response elements, CRE, recognized by transcription factors that are activated by the adenylate cyclase signal transduction pathway (8). The power of the assay is demonstrated by the distinct pharmacological profiles obtained for each receptor subtype. Furthermore, the assay is suitable to be used in automated screenings aimed at the development of ligands with greater selectivity for the different adenosine receptor subtypes.

MATERIALS AND METHODS

Plasmid constructions. The *SalI-XbaI* fragment containing the cDNA of the human A1 adenosine receptor (9) was subcloned into the *XhoI-XbaI* site of the eukaryotic expression vector pAD-CMV1 (10), which contains a dihydrofolate reductase (DHFR) minigene as a selectable marker. The resulting plasmid was named pWS1258E..
A *HindIII-BamHI* fragment containing the cDNA coding for the human adenosine A2a receptor (11) was inserted into the expression vector pAD-CMV1, resulting in plasmid pWS1253E. Insertion of a *PvuII* fragment containing the coding region of the human adenosine A2b receptor (12) into the *EcoRI* site of pAD-CMV2 (9), yielded plasmid pWS1247E. Plasmids used for transfections were purified using Quiagen columns (Diagen GmbH).

Cell transfections. The reporter cell line CHO C6-13 (8) was transfected by electroporation with 20 μg of *FspI* linearized plasmids pWS1258E, pWS1253E or pWS1247E, respectively, and selected for DHFR expression in nucleotide free MEM α^- medium supplemented with 10% dialyzed fetal calf serum and the neomycin analog G-418 (700 μg/ml). Electroporation conditions were as described by Himmler et al. (8).

Luciferase assays. $25x10^3$ cells/well were seeded into 96-well microtiter plates, light impermeable (Microlite, Dynatech Laboratories Inc, Chantilly, VA), and incubated overnight at 37°C. Cells were induced by the addition of adenosine agonists to either forskolin (2 μM) stimulated cells, in the case of the A1 receptor, or to unstimulated cells, in the case of the A2 receptors. After 4 h incubation at 37°C luciferase activity was measured in a 96-well luminometer model ML-1000 (Dynatech) as described by Weyer et al. (13). For antagonistic studies, drugs were added to the cells prior to agonist stimulation. Data were fitted to a sigmoidal curve by non-linear regression analysis using the program GraphPAD (San Diego, CA). The agonists and antagonists tested were purchased from Research Biochemical Inc. (Natick, MA).

RESULTS AND DISCUSSION

The opposite coupling of A1 and A2 adenosine receptors to adenylate cyclase is well documented (1-5). We took advantage of the cAMP sensitivity of the CHO reporter cell line C6-13 to demonstrate that the negative and positive coupling of the A1 and A2 receptors, respectively, to adenylate cyclase, and thus the activation state of the receptor, could be easily and efficiently monitored by bioluminescence. The stably transfected reporter cell line CHO C6-13 has been derived from the DHFR-deficient chinese hamster ovary cell line CHO-DXB11 by random integration of a reporter construct containing six CREs upstream a minimal β-globin promoter (8). Stimulation of

adenylate cyclase with 20 μM forskolin in the reporter cell line results in 20 to 30-fold induction of luciferase activity (8). This reporter cell line was further transfected with the human adenosine A1, A2a and A2b receptors encoded in the expression plasmids pWS1258E, pWS1253E, pWS1247E, respectively. Clones expressing the various recombinant receptors were screened by measuring luciferase activity after induction with a selective ligand. Addition of adenosine (1 μM) to forskolin stimulated cells was used to screen for the A1 receptor, whereas addition of adenosine (1 mM) to unstimulated cells was used to screen for the A2 receptors. A total of 800 clones were tested, 400 for the A1, and 200 for each of the A2a and A2b adenosine receptors. Clones CHO/A1-B1835, CHO/A2a-11H and CHO/A2b-9/11 were selected on the bases of their best ligand-dependent modulation of luciferase activity.

Functional characterization of the human A1 receptor in CHO cells

Cells stably transfected with the adenosine A1 receptor responded to adenosine receptor agonists with a quantitative decrease in luciferase expression, an indication that the negative coupling to the endogenous G_i proteins is functionally operative. Fig. 1A shows the dose-dependent inhibition by adenosine agonists of forskolin-induced luciferase activity in CHO-A1/B1835 cells. Addition of the A1 specific ligands, CCPA, CPA and R-PIA, at 10^{-5} M, resulted in 85-90% inhibition of forskolin-induced luciferase activity (EC_{50} values in the lower nanomolar range). Similar results were obtained when other A1 specific agonists such as CHA were tested (results not shown). In contrast, addition of the A2a specific ligand, CGS21680 at 10^{-5} M, resulted only in 60% inhibition (EC_{50} in the micromolar range). The inhibitory effect on the nonselective ligand, NECA, is also shown since it has been instrumental in the distinction of the A2a and A2b receptor subtypes (see below). The rank order of potency for inhibition of forskolin stimulation of adenylate cyclase

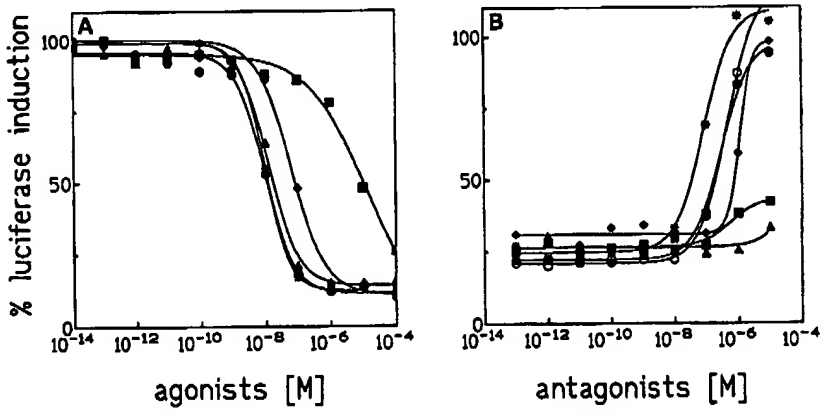

Fig. 1. Luciferase response curves for adenosine agonists and antagonists of forskolin-stimulated CHO cells stably transfected with the A1 adenosine receptor. A. Inhibition of forskolin-stimulated luciferase activity by increasing concentrations of agonists (●, CCPA; ▲, R-PIA; ◆, NECA; ■, CGS; ✱, CPA). B. Inhibition of the agonist-induced response by increasing concentrations of antagonists (●, PACPX; ▲, DMPX; ◆, 8-PT; ■, AM; ✱, XAC; O, CTP). Antagonists were added prior to stimulation with 0.1 μM CCPA. Results are shown as percentage of maximal luciferase induction observed with 2μM forskolin. Data points represent the mean of quadruplicate determinations. Shown are the results of one of three independent experiments.

in the described system is: CCPA=CPA>R-PIA>NECA>CGS. The agonist dependent inhibition of luciferase activity on forskolin-stimulated cells could be reversed, in a dose-dependent manner, by treatment with different antagonists (Fig. 1B). In the cases of potent A1 selective antagonists, such as PACPX and XAC, luciferase expression was restored to the levels obtained by direct activation of adenylate cyclase with forskolin. In contrast, addition of the A2 specific antagonist, DMPX, had little effect on luciferase activity, which remained at the levels dictated by the agonist. As certain xanthine derivatives are known to inhibit phosphodiesterase (PDE) activity, and thus to cause an increase in intracellular cAMP, we assayed all the antagonists used in this study for their ability to induce luciferase activity in the reporter cell line (no receptor). In most of the cases, we found no significant interference due to inhibitory effects on PDEs over the range of concentrations tested, 10^{-4} to 10^{-9} M (results not shown). Some of these novel xanthines have been reported to be about 100-1000-fold more potent as adenosine receptor antagonists than as PDE inhibitors (14). However two A1 selective antagonists, CTP and DPX, and DPMX, A2 selective; were able to induce luciferase expression when assayed at concentrations higher than 1 μM. This effect is additive in the presence of the receptor, resulting in slightly inflated luciferase induction rates for the highest values of the dose-response curves (results for CTP shown in Fig. 1B). DPX was found to be the strongest inhibitor of PDE when tested in our system. The order of potency for the antagonists shown in Fig. 1B is: XAC>PACXC>CTP>8-PT>AM>DMPX. Thus, the deduced potency order of the agonists and antagonists tested agrees with previously reported data for the human A1 receptor obtained from tissue preparations (1, 3-4) and transfected CHO cells (9), based on classical binding assays.

Functional characterization of human A2a and A2b receptors in CHO cells

Cells stably transfected with adenosine A2 receptor expression constructs responded with a measurable induction of luciferase activity, upon stimulation with specific ligands. Fig 2A shows the dose-dependent response of CHO/A2a-11H cells to several adenosine agonists. The highest induction of luciferase expression was obtained with the A2a selective agonist CGS21680 (EC_{50} in the low nanomolar range) and the lowest with CCPA, A1 selective. Adenosine was the least potent ligand tested (results not shown). The relative potencies for the agonists are: CGS21680> R-PIA=NECA>CCPA. Agonist induced luciferase expression could be blocked in a dose-dependent manner by addition of known adenosine antagonists (Fig. 2B). The rank order of potency for the antagonists tested is: DMPX>AM>ADSPX>DPMX.

A similar set of experiments was carried out with cells stably transfected with the low affinity receptor subtype, CHO/A2b-9/11. The results obtained are shown in Fig. 3A and 3B. Agonist-activated A2b receptors induced luciferase expression with the potency order of CPCA>NECA> R-PIA=CCPA>CGS21680. More significantly, the response of the CHO/A2b-9/11 cell line to the A2a receptor selective agonists CGS21680 in terms of luciferase activity, is about 1000-fold less than that of the CHO/A2a-H11 cell line (Fig. 2A), and the response to NECA 100 fold-less. In contrast, no marked differences were found for the antagonists; a result that merely reflects the fact that no A2 selective antagonists have yet been identified (3). Thus, agonists and antagonists induced luciferase activity with an order of potency consistent with published data for the human

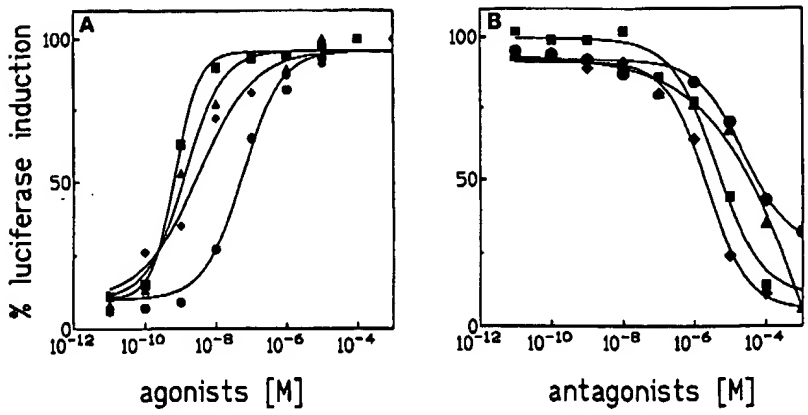

Fig. 2. Luciferase response curves for adenosine agonists and antagonists in CHO cells stably transfected with the A2a adenosine receptor. A. Induction of luciferase activity by increasing concentrations of agonists (●, CCPA; ▲, R-PIA; ◆, NECA; ■, CGS). B. Inhibition of agonist-induced luciferase activity by increasing concentrations of antagonists (●, DPMX; ▲, ADSPX; ◆, DMPX; ■, AM). Antagonists were added prior to stimulation with 1 mM adenosine. Results are shown as percentage of maximal luciferase induction and reported as described in Fig. 1.

A2a and A2b adenosine receptors obtained from tissue preparations and from stably transfected cell lines (11-12).

Conclusions

Stable expression of cloned receptors in mammalian host cell lines is becoming the method of choice in the study of receptor function and the search for novel ligands. Clonal cells expressing a

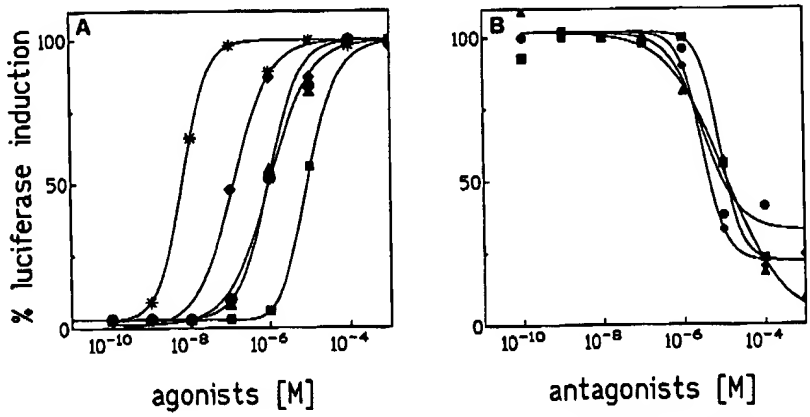

Fig. 3. Luciferase response curves for adenosine agonists and antagonists in CHO cells stably transfected with the A2b adenosine receptor. A. Induction of luciferase activity by increasing concentrations of agonists (●, CCPA; ▲, R-PIA; ◆, NECA; ■, CGS; ✱, CPCA). B. Inhibition of agonist-induced luciferase activity by increasing concentrations of antagonists (●, DPMX; ▲, ADSPX; ◆, DMPX; ■, AM). Antagonists were added prior to stimulation with 1 mM adenosine. Results are shown as percentage of maximal induction and reported as described in Fig. 1.

particular receptor, or receptor subtype, have been used for the pharmacological analysis of receptor agonists and antagonists by ligand binding and effector modulation assays (9, 11-12). Binding assays, however, are not functional assays. High affinity binding does not necessarily correlate with eliciting a functional effect, thus with productive binding. Furthermore, non-competitive or allosteric effectors cannot be detected. Effector modulation assays better reflect function, but generally require cumbersome procedures for measuring parameters such as cAMP accumulation, IP_3 turnover, intracellular Ca^{2+} concentration, or electrophysiological changes (7). A recent improvement in the area of functional receptor assays has been the development of transcription assays based on the expression of a reporter gene placed under the control of DNA regulatory sequences activated upon ligand binding (8,13). We report here the pharmacological profile of the A1, A2a, and A2b, adenosine receptors derived from this type of assay. Induction of luciferase expression by known adenosine receptor agonists and antagonists occurred with a rank order of potency and affinity characteristics of each receptor. Cells transfected with the A1 receptor respond very modestly to stimulation with the A2a selective ligand CGS21680, and vice versa; cells transfected with the A2a and A2b receptor, elicit a week response upon stimulation with the A1 specific ligands CCPA. Our system, thus could greatly facilitate the search for new ligands in general, and the search for A2 selective antagonists (3) or the missing A2b specific ligand (12), in particular. As growing evidence points to the existence of A1 receptor subtypes (3-4), the CHO/A1-B1835 cell line provides a system to start addressing this question.

ACKNOWLEDGMENTS

We thank K. Watzer for excellent technical assistance; A. Himmler and T. Voss for comments on the manuscript, insightful suggestions and help with the graphic program; F. Libert, J. Shine and P. Czernilofsky for helpful discussions.

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